Effects of changes in structure of plasmid-encoded β-lactamases on the binding of the third generation cephalosporins

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Abstract

Until 1983 the third-generation cephalosporins (3GCs) were thought to be resistant to hydrolysis by all plasmid-mediated β-lactamases. By the end of the 1980s, however, transferable TEM- and SHV-*derived* β-lactamases, known as extended-spectrum β-lactamases (ESBL), that conferred resistance to the 3GCs, were regularly identified in clinical isolates.

The gene encoding TEM-E2 (*bla*T-E2), an ESBL identified in an isolate responsible for an outbreak of infection in a neonatal unit in 1982, was sequenced. As cloning of PCR-amplified DNA fragments proved difficult, a new technique for direct DNA-sequencing of PCR-amplified DNA fragments was developed. From the DNA sequences of *bla*T-E2$_{pUK721}$ generated by this method, a mutant adenine residue was identified at DNA residue number 692. pUK721 encoded both TEM-1 and TEM-E2, the latter enzyme being identical to the TEM-12 β-lactamase.

Silent mutations, or minimally important mutations, occur frequently with little functional consequence, but may prepare bacteria for a more drastic change when challenged with a 3GC: acquisition of a single innocuous mutation facilitates the occurrence of a more dangerous double mutation. Clavulanic acid, in combination with amoxycillin, has been shown to promote back-mutations in ESBL by a mechanism of gene duplication facilitated by intramolecular transposition.

Escherichia coli J62-2 (TEM-5$_{pCFF14}$) was subcultured in liquid media containing limiting concentrations of ampicillin or amoxycillin/clavulanic acid (ratio 2:1) for five days. The minimum inhibitory concentrations (MICs) of various β-lactam antibiotics revealed some strains with increased resistance to amoxycillin/clavulanic acid, ceftazidime and cefotaxime. Increased resistance corresponded to multiple β-lactamase expression (three enzymes with isoelectric points (pI) of 5.57, 5.5 and 5.25). Enzyme kinetics of each β-lactamase showed significant similarity to the

TEM-10 (pI 5.57), TEM-5 (pI 5.5), and TEM-12 (pI 5.25) β-lactamases; this suggested that these were the identities of the mutant β-lactamases. Southern blotting of restricted plasmid DNA showed that gene duplication had occurred to create the mutant plasmid, pUK3007, from the parent plasmid (pCFF14).

Strains of *E. coli* J62-2 containing the mutant plasmid (pUK3007) or the parent plasmid (pCFF14) were passaged in liquid media containing limiting concentrations of ampicillin or amoxycillin/clavulanic acid (ratio 2:1) for a further 20 day period. Analysis of passaged strains revealed that anomalous changes in the MICs of amoxycillin/clavulanic acid, cefotaxime and ceftazidime corresponded to the presence of one of two β-lactamases (pI 5.5, and 5.4). Each strain was shown to harbour a single plasmid, and Southern blotting showed the presence of one TEM β-lactamase gene or two TEM β-lactamase genes despite each strain expressing only a single β-lactamase. Enzyme kinetics of each β-lactamase confirmed that the novel enzymes were TEM-5 (pI 5.5) and TEM-1 (pI 5.4). The combination of amoxycillin with clavulanic acid promotes the selection of either a 'terminus' ESBL or a narrow-spectrum prototype enzyme within an individual strain.

I suggest the hypothesis that the evolution of the parent β-lactamase to ESBL can consist of both forward and backward mutations at well defined locations. My results have shown that amoxycillin in combination with clavulanic acid selected back mutations to TEM-1 or the intermediate ESBL. The back mutations were facilitated by gene duplication. The point-mutations occur in the TEM-gene during duplication of the gene. In a non-selective environment one of the duplicate genes is usually deleted.

Escape into the environment may result in challenge of the host organism with a penicillin and a β-lactamase inhibitor. Such a biological pressure would select against the ESBL, and may induce back mutations to the more innocuous enzymes, for example TEM-17 or SHV-3. These results would also help explain the evolution of such a diversity of "terminus" enzymes, such as TEM-3, TEM-5, TEM-9 and TEM-24, that essentially provide the same service to the host strain.

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

S.K. Du Bois

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I dedicate this thesis to my parents. In addition I should like to dedicate this thesis to the memory of my paternal grandmother and my great uncle, both of whom passed away during the course of my studies in Edinburgh.

Contents

Abbreviations

The common abbreviations used throughout the text of this thesis are listed alphabetically below. Standard amino acid abbreviations are listed in the table on page xvi, and the genetic code is given in the table on page xvii.

%C	total crosslinking monomer concentration per 100ml
%T	total monomer concentration per 100ml
%V/v	volume (ml) per 100ml
%W/v	weight (g) per 100ml
3GC	third generation cephalosporin
6-APA	6-aminopenicillanic acid
7-ACA	7-aminocephalosporanic acid
A	adenine
A_{260}	absorbance at $\lambda = 260$nm
A_{280}	absorbance at $\lambda = 280$nm
ABL	Ambler Class A β-lactamase
Amp	ampicillin
Amx	amoxycillin
bla	β-lactamase gene
*bla*T	TEM β-lactamase gene
bp	base pairs
BSA	bovine serum albumin
BSAC	British Society for Antimicrobial Chemotherapy
C	cytosine
Caz	ceftazidime
Cep	cephaloridine
cfu	colony forming units
Clav	clavulanic acid
Ctx	cefotaxime
dATP	2'-deoxyriboadenosine triphosphate
dCTP	2'-deoxyribocytidine triphosphate
dGTP	2'-deoxyriboguanosine triphosphate
DM	Davis & Mingioli minimal salts medium
DMF	*N,N,*-dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dTTP	2'-deoxyribothymidine triphosphate
EDTA	ethylenediaminetetraacetate
ESBL	extended-spectrum β-lactamase
g	acceleration due to gravity
G	guanine
his	histidine auxotrophy
i.m.	intramuscular injection
i.v.	intravenous injection

I_{50}	concentration required to inhibit 50% of enzyme activity
IEF	isoelectric focusing
IPTG	isopropyl-β-D-thiogalactopyranoside
IST	Isosensitest medium
ITU	Intensive Therapy Unit
Kan	kanamycin
kb	kilobases
k_{cat}	turnover number
K_m	Michaelis constant
λ	wavelength of light
λ DNA	lambda phage DNA
lac	lactose non-fermentor
lacZ	β-galactosidase gene
lacZ'	portion of the *lacZ* gene encoding α-protein
LB	Luria-Bertani medium
M.Wt.	molecular weight (mass)
mA	milliAmperes
MCS	multiple cloning site
MGT	DM medium plus glucose and thiamine
MIC	minimum inhibitory concentration
N	purine
Nal	nalidixic acid
Ncfn	nitrocefin
OD_{550}	optical density at λ = 550nm
OMP	outer membrane protein
ORF	open reading frame
p.o.	oral administration (*per oris*)
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PEG	polyethylene glycol
PenG	penicillin G (benzylpenicillin)
pI	isoelectric point
pro	proline auxotrophy
PVP	polyvinylpyrrolidone
R	resistant
Rif	rifampicin
RNase A	ribonuclease A
rpm	revolutions per minute
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate
SSB	single-stranded DNA binding protein
SSC	sodium chloride/*tri*-sodium citrate buffer
SSCP	single-stranded conformational polymorphism
T	thymine
TAE	Tris·acetate/EDTA buffer
TBE	Tris·borate/EDTA buffer
TE	Tris·Cl/EDTA buffer
TEMED	*N,N,N',N'*-tetramethyl-ethylenediamine
Tet	tetracycline
thi	thiamine auxotrophy
Tn	transposon
Tris	tris(hydroxymethyl)-methylamide
trp	tryptophan auxotrophy
UV	ultra-violet light
V	volts
V_{max}	maximum rate of hydrolysis
W	Watts
X-gal	5-bromo-4-chloro-3-indoyl-β-galactopyranoside
Y	pyrimidine

Amino acid	Three-letter abbreviation	One-letter abbreviation	Molecular weight
Alanine	Ala	A	89
Arginine	Arg	R	174
Asparagine	Asn	N	132
Aspartic acid	Asp	D	133
Asparagine or aspartic acid	Asx	B	-
Cysteine	Cys	C	121
Glutamine	Gln	Q	146
Glutamic acid	Glu	E	147
Glutamine or glutamic acid	Glx	Z	-
Glycine	Gly	G	175
Histidine	His	H	155
Isoleucine	Ile	I	131
Leucine	Leu	L	131
Lysine	Lys	K	146
Methionine	Met	M	149
Phenylalanine	Phe	F	165
Proline	Pro	P	115
Serine	Ser	S	105
Threonine	Thr	T	119
Tryptophan	Trp	W	204
Tyrosine	Tyr	Y	181
Valine	Val	V	117

Abbreviations & molecular weights of amino acids

First base of codon	Second base of codon	Third base of codon			
		G	A	C	T
G	G	Gly	Gly	Gly	Gly
	A	Glu	Glu	Asp	Asp
	C	Ala	Ala	Ala	Ala
	T	Val	Val	Val	Val
A	G	Arg	Arg	Ser	Ser
	A	Lys	Lys	Asn	Asn
	C	Thr	Thr	Thr	Thr
	T	Met	Ile	Ile	Ile
C	G	Arg	Arg	Arg	Arg
	A	Gln	Gln	His	His
	C	Pro	Pro	Pro	Pro
	T	Leu	Leu	Leu	Leu
T	G	Trp	UGA	Cys	Cys
	A	Stop (amber)	Stop (ochre)	Tyr	Tyr
	C	Ser	Ser	Ser	Ser
	T	Leu	Leu	Phe	Phe

The genetic code

Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; U, uridine. The three letter amino acid abbreviations are listed in the table on page xvi.

Publications & Presentations

PUBLICATIONS

MacDonald, A.A., P.A.C. Maple, C.C. Kibbler, R.C. George, A.P. Johnson, S.K. Du Bois, & S.G.B. Amyes (1991). Ceftazidime-resistant *Klebsiella pneumoniae*. *Lancet* **337**: 1609-1610.

Amyes, S.G.B., D.J. Payne, & S.K. Du Bois (1992). Plasmid-mediated β-lactamases responsible for penicillin & cephalosporin resistance. *Journal of Medical Microbiology* **36**: 6-9.

Johnson, A.P., M.J. Weinbren, B. Ayling-Smith, S.K. Du Bois, S.G.B. Amyes, & R.C. George (1992) Outbreak of infection in two U.K. hospitals caused by a strain of *Klebsiella pneumoniae* resistant to cefotaxime & ceftazidime. *Journal of Hospital Infection* **20**: 97-103.

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PRESENTATIONS

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Chapter 1

INTRODUCTION

1. PROLOGUE

There are primarily three mechanisms of β-lactam antibiotic resistance in bacteria:

- The target enzymes of β-lactams, collectively known as the penicillin binding proteins, can become insensitive to the antibiotic.
- The bacterial cell envelope can become impermeable to the β-lactam antibiotic through loss or mutation of certain outer membrane proteins (OMPs) or porins.
- Thirdly, and by far the most predominant method of β-lactam resistance, is the enzymatic destruction of the antibiotic. These enzymes are known generically as β-lactamases, but they are also called penicillinases if they only hydrolyse penicillins, or cephalosporinases if they only hydrolyse cephalosporins.

β-lactamases confer resistance to penicillins, cephalosporins and related β-lactam antibiotics by their ability to bind and hydrolyse these drugs before they reach their target sites. In bacteria these targets are known as penicillin-binding proteins (PBPs), and in Gram-negative bacteria are located on the inner membrane of the cell envelope. The β-lactamases of bacteria are cell bound, periplasmic, or secreted. In Gram-negative organisms the β-lactamases are located in the periplasm, between the outer

layer and the membrane, ready to intercept incoming β-lactam molecules. The β-lactamases of staphylococci and enterobacteria are the most studied of this class of enzyme because of their obvious role in the emergence of resistance to β-lactam antimicrobials in the clinical setting; β-lactamases are produced by many other genera, including *Mycobacterium*, *Legionella*, *Actinomyces*, and *Nocardia* [1]. Certain mammalian cell enzymes have also been shown to exhibit some β-lactamase function, for example renal dehydropeptidases [1].

Although many β-lactamases of clinical importance are encoded by genes on the bacterial chromosome, the most prevalent and successful resistance genes are carried by plasmids and usually within transposons. Unlike their chromosomally-encoded counterparts, the plasmid-mediated enzymes in Gram-negative bacteria are usually produced constitutively so that any change in the ability to confer resistance will probably stem from changes in the structure of the molecule rather than changes in expression of the gene.

The discovery and evolution of β-lactamases has paralleled the discovery and clinical use of β-lactam antimicrobial agents. The plasmid-mediated TEM- and SHV-*derived* extended-spectrum β-lactamases (ESBL) have evolved over a short period of time from the TEM-1 and SHV-1 β-lactamases. The plasmid-mediated TEM- and SHV-*type* β-lactamases are the most common mechanism of resistance to the β-lactam group of drugs in Gram-negative bacteria [2, 3]. These two ubiquitous enzyme types have a broad spectrum of activity and preferentially hydrolyse the penicillins together with some first- and second-generation cephalosporins. The ESBL, the substrates of which include the so called 'β-lactamase-stable' third-generation cephalosporins (3GCs), have emerged in clinical bacteria on every continent, and have become an enormous clinical threat to this class of pharmaceutical. The ESBL that are appearing sporadically are a response of the bacteria to treatment of infections by the 3GCs, ceftazidime and cefotaxime.

The β-lactam antibiotics have played a key role in reducing the impact of life threatening diseases. Serious infections, however, remain a major and potentially life-threatening problem among vulnerable patients. Intensive Therapy Units (ITUs), for example, provide an environment that is especially favourable to the development and spread of pathogens [4] and the ESBL.

2. THE HISTORY OF ANTIMICROBIAL AGENTS

Often traditional and folk remedies, many of which have been formulated through centuries of experience, can be shown to have a sound pharmacological basis for their ascribed action. A Mesopotamian pharmacopoeia, the earliest known and dating from about 2100BC [5], describes preparations for the control of sepsis, which work by osmotic effects. Honey was frequently used as a component of ancient nostrums for its anti-inflammatory and antimicrobial attributes. Osmotic desiccation can account for part of the observed efficacy, however, the non-monosaccharide components of honey have a more direct antimicrobial action [5]. In recent years, sugars, in the form of granulated sugar in combination with débriding agents, have had a resurgence in popularity for the treatment of wound infections [6-8].

Substances with better and more efficient antimicrobial agents have always been sought, therefore, even the most unlikely substances have been investigated. Fungi have been used in herbal medicine for many hundreds of years, partly for their antimicrobial action. The therapeutic use of hyssop by ancient civilisations may be significant when considering that the first description of *Penicillium notatum* followed its isolation from this small bushy aromatic herb [9]. Thomas Phayre's 'Boke of Chyldren' (1545), however, probably contains the first specific reference for the therapeutic use of a fungus for the treatment of bacterial infections. Further references for the use of fungi from various sources were made at intervals until the latter half of the 19th century [10, 11], when scientists began to investigate the attributed actions of moulds and fungi more thoroughly.

John Burdon Sanderson was the first to note, after a series of microbiological experiments in 1870, that bacteria did not grow in media with visible 'penicillum' mould growth. He misinterpreted the results and did not suspect that the mould itself had an antibacterial action. Lister in some of his pioneering bacteriology experiments, prompted by Sanderson's publications in 1871, also noted that *Penicillium* could inhibit the growth of some bacteria, but fully recognised the inhibitory effect of the mould on bacteria. In a letter in 1872 Joseph Lister expressed the intention to use *Penicillium glaucum* therapeutically in humans, and there is some evidence that he did in 1884 [12]. Several other workers, notably William Roberts, John Tyndell and Thomas Henry Huxley, also contributed to the pioneering work on *Penicillium* during

this decade. It was not, however, until the detailed report published by Fleming in 1929 on the antibacterial effects of *Penicillium notatum* [13], that Chain *et al.* were prompted to re-examine the phenomenon [14].

Paul Ehrlich's work in the first decade of this century must also be mentioned. Almost all the concepts which have governed subsequent work on synthetic antimicrobial agents were proposed by Ehrlich during his studies. Ehrlich believed that infectious disease could be treated with synthetic chemicals. He proposed that if enough variations of a compound group were synthesised, some would be made that would have sufficiently low toxicity to be safe for use as chemotherapeutic agents. A massive search for an organo-arsenical compound for the treatment of syphilis ensued, leading to the discovery of salvarsan in the 1900s [15]. Salvarsan, and its derivative neo-salvarsan, together with bismuth therapy were the standard treatment for syphilis until the introduction of penicillin in the 1940s.

3. THE DISCOVERY & DEVELOPMENT OF PENAMS

The discovery of penicillin is credited to Alexander Fleming, who noted in 1929 that a culture of *Penicillium notatum* inhibited the growth of *Staphylococcus aureus* [13]. Several other scientists, as described above, over the previous 60 years had also observed the antibacterial activity of *Penicillium* moulds. Fleming was the first, however, to realise that the inhibition resulted from the action of a small molecular mass compound produced by the mould, and suggested its use as an inhibitor in the isolation of certain types of bacteria, especially *Haemophilus influenzae*. He also noted that broth containing the substance, which he called penicillin, when injected into animals was no more toxic than an injection of plain broth. Fleming suggested that penicillin could be used as an antiseptic. The unstable nature of penicillin, however, thwarted early attempts at purification and hence prevented exploitation.

The clinical success of the sulphonamides [16, 17] renewed scientific interest in the chemotherapy of bacterial infections and stimulated Florey and Chain, in 1939, to re-examine 'penicillin' [14]. Chain and co-workers managed, where others had failed over the previous decade, to isolate an impure but highly anti-bacterial penicillin powder [14, 18]. Early clinical trials of penicillin [19] demonstrated its effectiveness, and, because of World War II, stimulated perhaps the biggest chemical and biological

joint research programme ever undertaken between companies in the UK and the USA. The collaboration led, not only to a vast output of penicillin, but also the foundation of the modern pharmaceutical industry. Since the 1940s, penicillin has been widely used for the treatment of various infections, and numerous penams have been discovered or synthesised [20] (Table 1.1 page 6). The identification of the anti-bacterial activity of penicillin by Fleming instigated the discovery of one of the largest and most important classes of antimicrobial agents, known as the β-lactam antibiotics, which are unrivalled in the treatment of bacterial infections. Their only serious defect is their characteristic induction of immunological sensitisation in a small percentage (usually about 10%) of the population, a side effect which precludes their use in those affected.

3.1. THE NATURAL PENICILLINS

The original penicillins, isolated directly from mould fermentations, were mixtures of compounds with different side-chains. Addition of adjuncts to the fermentation ensured that the product was predominantly a single compound. Penicillin G (benzylpenicillin) was the parent drug of this family produced by the addition of phenylacetic acid to the mould growth medium [21]. The first successful variant, phenoxymethylpenicillin (penicillin V), was obtained by adding phenoxyacetic acid to the medium rather than phenylacetic acid [22], although many compounds were tried as adjuncts. The main advantage of the side-chain modification of penicillin V over penicillin G was the improvement in acid stability. The ready inactivation of penicillin G at low pH limited its usefulness when given orally. Phenoxymethylpenicillin thus improved the reliability of oral administration. The early penicillins were very active against Gram-positive bacteria and gave excellent results in streptococcal and staphylococcal infections, and in pneumonia. Penicillin G and V are much less active against the more typical Gram-negative bacterial infections, except *Neisseria gonorrhoeae*. A tribute to the longevity of these compounds is that both penicillin G and penicillin V are still used regularly in clinical practice more than half a century after their introduction into medicine.

3.2. PENICILLINASE-RESISTANT PENICILLINS

Penicillinase, an enzyme which could inactivate penicillin by hydrolysis, was identified in *Escherichia coli* in 1940 by Abraham & Chain [23], the same year as the initial reports of the therapeutic potential of penicillins were published [13]. Although the discovery of penicillinase was an important consideration in the development of the β-lactams, the increased use and success of penicillins in the treatment of staphylococcal infections between 1945 and 1960, led to the emergence of penicillinase-producing staphylococci that rendered the natural penicillins ineffective. Barber's observation, in 1949 [24], that penicillin-sensitive

Type of penam	Generic name
Natural Penicillins	Penicillin G (benzylpenicillin) Phenoxymethylpenicillin (penicillin V) Phenoxyethylpenicillin (phenethicillin)
Penicillinase-resistant penicillins	Methicillin Oxacillin Nafcillin Cloxacillin Dicloxacillin
Aminopenicillins	Ampicillin Amoxycillin Bacampicillin
Carboxypenicillins	Carbenicillin Ticarcillin Temocillin
Ureidopenicillins	Mezlocillin Azlocillin Piperacillin
Amdinopenicillins	Amdinocillin Pivamdinocillin Foramdinocillin
Penicillin plus β-lactamase inhibitors	Amoxycillin-clavulanic acid Ticarcillin-clavulanic acid Ampicillin-sulbactam Piperacillin-tazobactam

Table 1.1 Classification of the penicillins

strains could acquire resistance after contact with penicillin-resistant strains led to the discovery of penicillinase encoding plasmids in Gram-positive bacteria [25]. Prior to this the transferable R-factors had only been observed in Gram-negative bacteria [26].

Enzymatic removal of the 6-β-benzyl side-chain of penicillin G allowed the isolation of the nucleus 6-aminopenicillanic acid (6-APA) (Figure 1.1), which could subsequently be modified by chemical means to create various semi-synthetic derivatives [27]. Development of semi-synthetic penicillins with an acyl side chain that sterically inhibited the action of penicillinase ensued. Methicillin was the first of this group, followed by, among others, oxacillin and the oral drug cloxacillin. These penicillins were widely used as the first line therapy of staphylococcal infections until the emergence of Methicillin-resistance *Staph. aureus* (MRSA) in the 1980s (for a recent review see Mulligan *et al.* [28]). Penicillinase-resistant penicillins have activity against the species *Streptococcus* and *Staphylococcus* but, unfortunately, little activity against Gram-negative organisms.

3.3. EXTENDED-SPECTRUM PENICILLINS

Increasing the antibacterial spectrum of penicillin derivatives has been the aim of antimicrobial research for many years. The need for penams with activity against Gram-negative bacteria prompted further modification of the 6-APA nucleus. The aminopenicillins were the first new group to be produced, followed by some

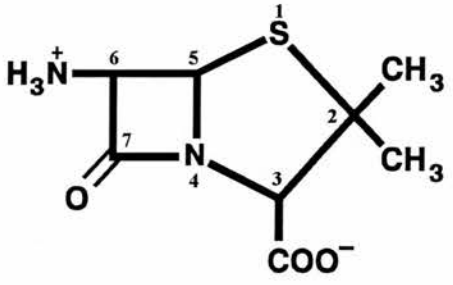


Figure 1.1: 6-Aminopenicillanic acid (6-APA)

Numbering of atoms starts from sulphur of five-membered thiazolidine ring (top right); continues clockwise to four-membered β-lactam ring through bridgehead nitrogen and tetrahedral carbon atoms (N-4 and C-5 respectively).

carboxypenicillins and ureidopenicillins. These classes of penicillin are not resistant to staphylococcal β-lactamases but are resistant, in variable degrees, to the chromosomally-encoded β-lactamases of Gram-negative bacteria. Improved antimicrobial activity is derived from increased penetration through the outer membrane of Gram-negative organisms and greater affinity for the targets of penicillin, the PBPs.

3.4. THE AMDINOPENICILLINS

Amdinocillin, another 6-APA derivative but with a 6-β-amidino side-group, has the unique property of binding preferentially to penicillin-binding protein type 2 (PBP-2), in contrast to other penicillins that bind to PBP-1 and PBP-3. In addition, amdinocillin is hydrolysed slowly by β-lactamases.

3.5. PENICILLINS PLUS β-LACTAMASE INHIBITORS

Further development of the penicillins has been impeded by the limitation of possible modifications of the 6-β-acyl side-chain, unlike the cephalosporins which can be modified at the 7-carbon (sterically equivalent to C-6 of 6-APA) and the 3-carbon of the 7-aminocephalosporanic acid (7-ACA) nucleus (Figure 1.3 page 10). Variation of the 6-acyl side-chain is limited by the stability, spectrum of activity, β-lactamase susceptibility, pharmacokinetics, and toxicity of the resultant penicillin. Hence, because of these limitations new strategies were explored.

The use of an inhibitor, with high-affinity and irreversible binding to bacterial β-lactamase, was introduced after the discovery of the β-lactamase inhibitor clavulanic acid [29, 30] (Figure 1.2 page 9) and provided an opportunity to co-administer a β-lactam with β-lactamase-susceptible penicillins such as amoxycillin, or ticarcillin. Other inhibitors have subsequently been discovered, for example, sulbactam and tazobactam, and used in combination with penicillins: ampicillin and sulbactam; piperacillin and tazobactam. Combinations of penicillins with β-lactamase inhibitors can prevent the most common method of bacterial resistance to penicillins; hydrolysis of β-lactams by β-lactamases.

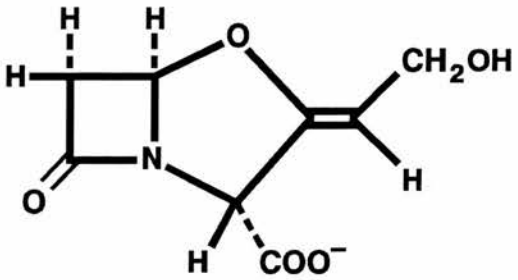


Figure 1.2: Clavulanic acid

Clavulanic acid is an *exo*-double bond isomer of the 1-oxa-2-penems. The sulphur atom of the five-membered thiazolidine ring of 6-APA has been replaced with an oxygen and the dual 2-methyl groups substituted with a hydroxyethylidene side-chain

4. THE DISCOVERY & DEVELOPMENT OF CEPHEMS

An antibiotic producing strain of *Cephalosporium acremonium* was isolated from a sewage outflow in Sardinia by Guiseppi Brotzu in 1945 [31] and he had some success, with crude preparations from this organism, against bacterial infections. Brotzu sent a culture of the mould to Sir Howard Florey in Oxford where the first substances were isolated from the organism. These compounds were a series of acidic steroids, collectively called cephalosporin P, that only had activity against Gram-positive bacteria [32]. The search for a second antibiotic isolated penicillin N (initially called cephalosporin N), closely related to the penicillins in structure [33], with activity against both Gram-positive and Gram-negative bacteria [34]. The antibacterial action of penicillin N was undoubtedly the cause of the antibiotic activities observed by Brotzu [35]. A small amount of a third antibiotic was observed during the analysis of penicillin N. The third compound, named cephalosporin C, possessed a β-lactam ring, but not the five membered thiazolidine ring characteristic of the penams [36]. Cephalosporin C was observed to have similar antibacterial activities to penicillin N, but was resistant to hydrolysis by penicillinase, hence provided potential clinical interest.

The isolation of the cephalosporin C nucleus, 7-amino-cephalosporanic acid (7-ACA), in the early 1960s [37] (Figure 1.3 page 10), has enabled the development of compounds that could be given orally, and that exhibit high activity against a range of penicillin resistant Gram-negative bacteria.

Prior to 1975 only a few cephalosporins were available for clinical use to the medical profession and it was relatively easy to distinguish between the compounds on the basis of antibacterial activity and pharmacology [38]. The production of successive 'generations' of cephalosporins since 1975 has suggested that each new group has provided general advances in every aspect of the cephems properties. A summary of the three recognised generations is given in Table 1.2 (page 11).

4.1. First-generation cephalosporins

Many of the first semi-synthetic cephalosporins are analogous in structure to the successful penicillins [39], and were highly active against a number of Gram-positive species at the time of their introduction. Although the first-generation cephalosporins showed significant activity against some Gram-negative bacteria, including various *Salmonella*, *Shigella*, and *Klebsiella*, their value for the treatment of a number of Gram-negative mediated infections was limited, and many of them were still susceptible to the TEM- and SHV-*type* of β-lactamases.

4.2. Second-generation cephalosporins

Modification of the side chains (C-3 and C-7) around the 7-ACA nucleus generated cephalosporins that were active against bacteria which had shown resistance to their predecessors. As the second-generation cephalosporins, in turn, fell foul of novel β-lactamases, compounds with a wider spectrum of activity and high stability against β-lactamase degradation were sought.

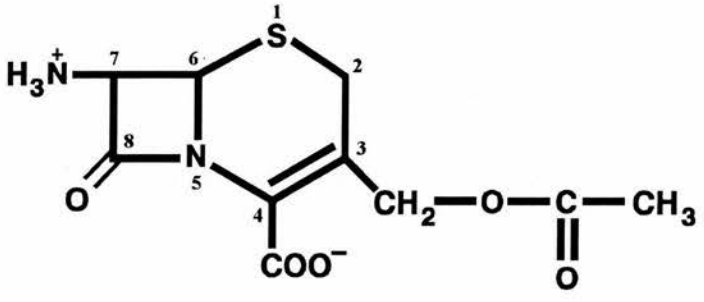


Figure 1.3: 7-Aminocephalosporanic acid (7-ACA)

Six-membered dihydrothiazine ring equivalent to the five-membered thiazolidine ring of 6-APA. Substitutions at positions 3 and 7 of 7-ACA are sterically equivalent to positions 2 and 6 of 6-APA.

Type	Generic name	Route†
First-generation	Cefaclor	p.o.
	Cefadroxil	p.o.
	Cefazolin	i.m., i.v.
	Cephalexin	p.o.
	Cephapirin	i.m., i.v.
	Cephadrine	i.m., i.v., p.o.
Second-generation	Cefamandole	i.m., i.v.
	Cefmetazole	i.v.
	Cefonicid	i.m., i.v.
	Ceforamide	i.m., i.v.
	Cefoxitin	i.m., i.v.
	Cefuroxime	i.m., i.v.
	Cefuroxime axetil	p.o.
Third-generation	Cefixime	p.o.
	Cefoperazone	i.m., i.v.
	Cefotaxime	i.m., i.v.
	Cefpodoxime proxetil	p.o.
	Ceftazidime	i.m., i.v..
	Ceftizoxime	i.m., i.v.
	Ceftriaxone	i.m., i.v.
	Cefepime	i.m., i.v.
	Cefpirome	i.m., i.v.

Table 1.2: Classification of the cephalosporins

† Route of administration: i.m., intramuscular; i.v., intravenous; p.o., oral

4.3. THIRD-GENERATION CEPHALOSPORINS

The most recent cephalosporins possess a broad spectrum of activity against Gram-negative bacteria, including some *Pseudomonas aeruginosa* strains. This group of cephalosporins were considered, at the time of their introduction, to be examples of the ultimate ability to overcome antimicrobial resistance, especially that mediated by TEM-1. Among these cephalosporins the increase in activity against Gram-negative bacteria is attributed to a modification of the side-chain, attached to the 7-carbon of the 7-ACA nucleus, containing a 2-aminothiazole group (e.g. cefotaxime, ceftazidime, ceftriaxone, ceftizoxime and cefmenoxime). These five cephalosporins also contain an *O*-substituted oxyimino group on the 7-β-acyl side-chain. Perhaps this group of cephalosporins can be epitomised by ceftazidime with a carboxylic alkoxyimino group, resulting in slow outer membrane

penetration but conferring a significant degree of anti-pseudomonas activity, and cefotaxime with a methoxyimino side-group, which gives the molecule faster penetration but little anti-pseudomonas activity. Ceftriaxone has similar properties to cefotaxime but has a much longer plasma half-life.

4.4. FOURTH-GENERATION CEPHALOSPORINS

Recently, the term 'fourth-generation cephalosporins' has been coined for the marketing of the previously designated third-generation cephalosporins cefpirome and cefepime. The fourth-generation cephalosporins have been identified by their 'bullet shaped' structure and a quaternary ammonium group, giving zwitterionic properties, at the 3-position of the cephem nucleus. Other cephalosporins (e.g. cephaloridine), however, also contain quaternary ammonium side-chains at the 3-position and are zwitterionic [20]. The bullet shape in combination with the positive charge at the 'point' of the bullet is believed to orientate the cephem when penetrating the outer membrane of the bacterium [40].

5. PENICILLIN & CEPHALOSPORIN BIOSYNTHESIS

Examples of the β-lactam class of antibiotics are produced by a wide variety of micro-organisms (Table 1.3 page 14), including some filamentous fungi, and a few Gram-positive and Gram-negative bacteria [41]. Eukaryotes and prokaryotes both produce penicillin and cephalosporin antibiotics by very similar biosynthetic pathways, a generic example of which is illustrated in Figure 1.4 (page 15). Various species of bacteria produce a range of cephalosporins or cephamycins, but none have been found to synthesise penicillins. In contrast, the fungi have only been found to contain genes for penicillin or cephalosporin biosynthesis. It is generally accepted that the genes associated with β-lactam biosynthesis, collectively known as the β-lactam synthetase genes, exist in gene clusters on the chromosome of the respective micro-organism, as first demonstrated by Malpartida & Hopwood [42].

5.1. THE ORIGIN OF THE β-LACTAM SYNTHETASE GENE

In general, the genes controlling successive steps in antibiotic biosynthesis, are clustered and there are no currently proven exceptions [43]. This is certainly true for the genes involved with β-lactam synthesis. Clustering of functionally related

genes implies that they have evolved as a unit, and enhances the probability that transfer of all the genes associated with biosynthesis occurs simultaneously [44]. This, together with high sequence similarity and narrow distribution of the β-lactam synthetase genes amongst micro-organisms, has led to a debate on the evolution of these genes. The currently accepted theory estimates that the genes transferred between species about 370 million years ago [45], after the eukaryote/prokaryote split (2 billion years ago [46]) but before the Gram-positive/Gram-negative split (1.5 million years ago [46]), rather than developing independently in parallel.

Bacteria possess a greater capacity to synthesise β-lactams than fungi, and are believed to be the donors in the ancient transfer event [47]. For transfer to occur the micro-organisms would have be in close contact, probably in the soil as all three participants (Gram-positive bacteria, Gram-negative bacteria, and fungi) are known to exist in this environment [48]. Conjugation in the soil environment has been demonstrated but is dependant on many factors, including geological factors and chemical interference from other soil micro-organisms [49].

5.2. THE ECOLOGY OF β-LACTAM PRODUCTION IN MICRO-ORGANISMS

Metabolism can be divided into two types: primary and secondary. Primary metabolic processes, essential for cell life, are generally far less complex than secondary metabolic pathways. Secondary metabolites, which include the antibiotics, are generally small molecular mass compounds, and are produced by a number of organisms including bacteria, plants and lower animals. Biosynthesis of secondary metabolites is not essential for the survival of the producing organism in a non-selective environment, but it has been proposed that they confer a selective advantage on the producing organism under certain conditions [44]. The chemical structures of secondary metabolites are responsible for their biological activity, and this activity is thought to confer a chemically-mediated defence mechanism on the host organism, analogous to the immune system of higher animals [44].

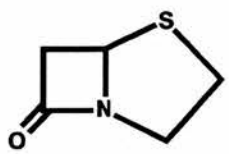
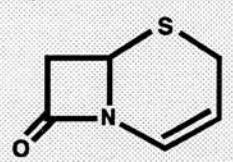
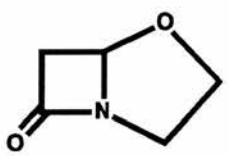
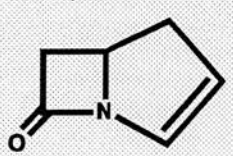
Class of β-Lactam	Fungi§	Bacteria§	
		Gram-positive	Gram-negative
Penam 	*Aspergillus* *Epidermophyton* *Malbranchae* *Penicllum* *Pleurophomopsis* *Polypaecilum* *Sartorya* *Trichphyton*		
Cephem 	*Anixiopsis* *Arachnomyces* *Cephalosporium* *Diheterospora* *Paecilomyces* *Scopularisiopora* *Spiroidium*	*Nocardia* *Streptomyces*	*Flavobacterium* *Lysobacter* *Xanthomonas*
Clavam 		*Streptomyces*	
Carbapenem 		*Streptomyces*	*Erwinia* *Serratia*
Monobactam 		*Nocardia*	*Acetobacter* *Agrobacter* *Chromobacter* *Gluconobacter* *Pseudomonas*

Table 1.3: Micro-organisms that produce β-lactam antibiotics

§ Only certain species within the micro-organisms listed make the indicated β-lactam antibiotic. After Aharanowitz, Cohen, & Martin, (1992) [41].

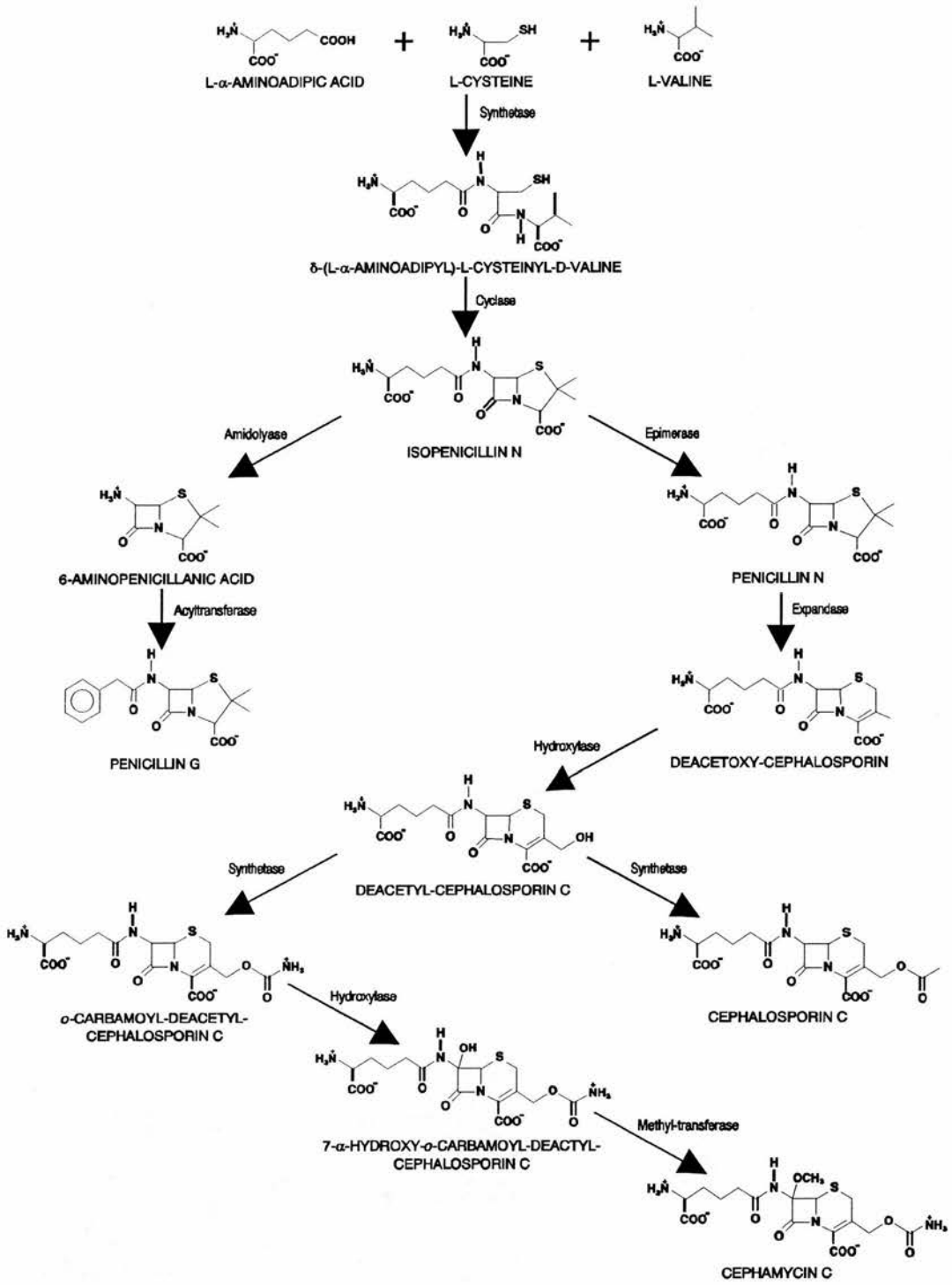


Figure 1.4: Generic pathway for the biosynthesis of β-lactam antibiotics

Many of the enzymes of the pathway are multi-functional; type of activity exhibited at each step is noted, not the specific enzyme. Modified from Aharanowitz, Cohen & Martin. (1992) [41].

Antibiotics are thought to have evolved to hinder the growth of other micro-organisms competing for the same nutrients, hence production would confer a selective advantage on the host organism [2]. Certainly, soil is estimated to harbour a large number of micro-organisms [48], and antibiotic production of some soil dwelling bacteria has been associated with vulnerable stages of the life-cycle of those organisms [50]. Hence, production of growth-inhibitors may be advantageous to the producer [44].

In order to avoid the potentially lethal effects of antibiotic synthesis, micro-organisms have had to develop mechanisms of defence against their own secondary metabolite production [2, 51, 52]. There are principally three methods of protection against intracellular metabolites during antibiotic biosynthesis [51]:

- Modification of the target-site to render it insensitive to a particular metabolite.
- Production as inert derivatives which are activated during or after export from the cell.
- Confinement of toxic products within discrete sub-cellular compartments during biosynthesis.

Obviously, although certain eukaryotes may exploit the third method, prokaryotes cannot. Self-defence in antibiotic-producing *Streptomyces* is well documented to occur by the former two mechanisms: target-site modification or metabolite inactivation [51-53]. Sequence similarities between the genes that encode enzymes for biosynthesis and antibiotic-inactivation within clusters in *Streptomyces* suggest that resistance has evolved from the biosynthetic genes [2, 43, 44]. Point mutations of the relevant DNA, facilitated by gene duplication, eventually created enzymes that modified the *Streptomyces* antibiotic streptomycin and its active-intermediates to a less active form [42, 44]. Resistance is, therefore, conferred on the host by an extra step in the synthesis of the secondary metabolite.

The genes encoding self-resistance are frequently interspersed into the biosynthetic/regulatory gene cluster, and often transcription of the proteins mediating resistance is part of the regulatory mechanism of biosynthesis [54]. In these pathways, self-resistance is established preceding antibiotic production. Integration of DNA encoding self-resistance into the gene cluster also guarantees

co-transfer between micro-organisms and ensures resistance in the recipient. The observation that antibiotic biosynthetic gene clusters appear to have little DNA unassociated with biosynthesis, regulation or resistance [44], adds weight to the theory that antibiotic production would confer a selective advantage on the producing-organism in a competitive environment.

In conclusion; antibiotics are thought to have evolved in the soil environment to provide a chemical defence mechanism to the host. Organisms, which produce these secondary metabolites, have also evolved an integral resistance mechanism to prevent the fatal effects of self-intoxication, often by modification of the biosynthetic enzymes. Resistance mechanisms associated with antibiotic production, however, are not the only sources of clinical resistance genes, other mechanisms appear to have evolved separately [2].

6. THE STRUCTURE & ACTIVITY OF THE β-LACTAM ANTIBIOTICS

The β-lactam antimicrobial agents are the most extensively studied and are, consequently, the most structurally varied class of drug. This is partly because of the ease with which the biological activities of the β-lactams can be varied by modification of the penicillin and cephalosporin rings. Each of the seven or eight positions on the bicyclic 6-APA or 7-ACA nuclei have undergone modification with the exception of the 4(5)-bridgehead nitrogen atom. Not only have the type of substituents of the nuclei been altered, but also the configuration of ring substituents, size and atom content of the rings, and position and configuration of ring junctures, in the search for better antimicrobial, pharmacokinetic, and pharmacological properties of the β-lactam.

6.1. THE CONSENSUS STRUCTURE OF THE β-LACTAM ANTIBIOTIC

β-lactams usually have a fused-ring at the heart of their structure, but this is not essential. The four membered lactam ring, present in all this class of antimicrobial agent, however, is essential for antibiosis. Except for the monobactams and the nocardicins, the β-lactam ring is fused through the bridgehead nitrogen atom (N-4(5)) and adjacent tetrahedral carbon atom (C-5(6)) to a second five or six

membered ring. Stereochemistry of the bicyclic nucleus around the β-lactam rings of the penicillins and cephalosporins is the same. The asymmetric centres at C-5 and C-6 of the penicillins correspond to C-6 and C-7 of the cephalosporins. Nearly all β-lactam compounds have a carboxyl group attached to the C-3(4) (adjacent to the bridgehead nitrogen) that is on the α-side of the ring system. Even the nocardicins, which lack the fused-ring structure, possess a carboxyl group that is congruent with the stereochemistry of the penams and cephems.

6.2. STRUCTURAL ASPECTS OF ACTIVITY

The activity of the β-lactam antimicrobial agents is the result of the inhibition of the growth of the bacteria, and is usually bactericidal. Bacteriostasis and/or bactericide is achieved through the interaction of the β-lactam compound with the complex dynamic processes involved in bacterial cell-wall synthesis. In both the penicillins (Figure 1.5a: X = S, R_2 & R_3 = H) and cephalosporins (Figure 1.6a page 21: Z = S, R_6 = H) a 6(7)-β-acylamino side-group (or equivalent) is necessary for antibiosis of bacteria. Manipulation of this group has created the most clinically useful agents in this class of drugs [55].

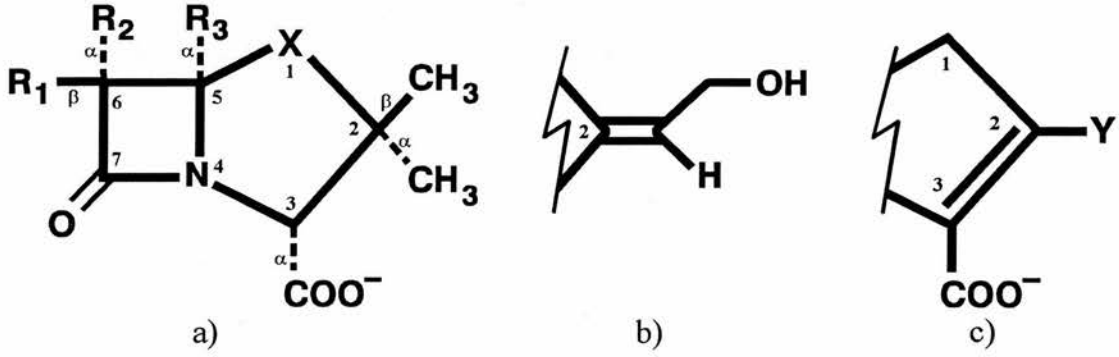


Figure 1.5: Generic structure of β-lactam antimicrobial agents I: penams, clavams & penems

Nomenclature: numbering of ring atoms according to that of 6-APA; α, denotes side-group "below" the plane of the fused-ring (i.e. into page); β, denotes side-group "above" the plane of the fused-ring (i.e. out of page). a) Penams (X = S), penems (X = S, C_2-C_3 double bond), sulfones (X = S-O_n), b) Clavams (X = O) c) Carbapenems: thienamycins, olivanic acids.

The prerequisite for the presence of a 6(7)-β-acylamino side-group (Figure 1.5: R_1 = -CONH-) for antibacterial activity is illustrated by the absence of antibiosis of clavulanic acid (see Figure 1.2 page 9 and Figure 1.5b) which lacks this side-group. The high antibacterial activity of the thienamycins (Figure 1.5c: groups R_2 or R_1 = 6-α- or 6-β-hydroxyethyl side-chains respectively), therefore, is unexpected [55]. Good antimicrobial activity is thought to result from optimal chemical reactivity of the β-lactam ring. Penicillin and cephalosporin fused-ring systems require additional activation through the 6(7)-β-acylamino substitution to raise the level of antibacterial activity of the compound [56], however, this activation has also been achieved with a 6-β-amidino group (Figure 1.5a: R_1 = -C(*R*)=N-) [57].

Unless a compound retains *in vivo* antimicrobial activity, and the appropriate pharmacokinetic and pharmacological properties, after introduction of a modification, the drug will not be useful clinically.

THE PENICILLINS.

A majority of the clinically useful penicillins are 6-β-acylamino substituted, and of those high antibacterial activity is narrowly confined to additional substitution with carboxamides [58-63]. As a consequence, only minor differences between analogues are observed [64]. Most 6-β-side-groups are derived from *mono-* or *di-*substituted acetic acids [21, 22, 65-68] which have improved acid stability, oral absorption, resistance to staphylococcal β-lactamases, and broadened Gram-negative activity. Other side-groups can considerably change activity: the 6-β-amidinopencillins, for example, have very low Gram-positive activity, but are very active against strains of *E. coli*, including those expressing plasmid-mediated β-lactamases (partly explained by low enzyme affinity) [57, 69].

Staphylococcal penicillinase susceptibility has principally been reduced by increasing steric hindrance around C-6 or by the introduction of an extra bond into the five-membered ring, for example, the 6-membered dihydrothiazine ring of the cephalosporins. Unfortunately, many of the changes in penicillin structure have had a deleterious effect on one or more of the desired properties. For example, penicillinase-resistant penicillins (e.g. methicillin, oxacillin) are virtually devoid of Gram-negative activity at the expense of good Gram-positive activity and penicillinase-resistance [64].

In general, substitution of the highly ionic acidic groups or hydrophilic groups on the α-carbon of the 6-β-side chain decreases the minimum inhibitory concentrations (MICs) towards Gram-positive bacteria but raises the anti-Gram-negative effect and extends the spectrum of activity in this genera [55]. Inclusion of alkyl groups into the 6-β-side-group can also help to optimise Gram-negative activity, but unsaturated 6-β-groups will improve Gram-positive activity, as does the introduction of heterocyclic or aromatic rings (usually at the expense of Gram-negative activity). Hetero atoms in the cyclic analogues have variable effects, but properly placed can invert the ratio of Gram-positive to Gram-negative activity. Also, *para*-hydroxy substitutions of 6-β-aromatic groups can increase oral absorption as does the formation of pro-drugs through conjugative esterification of various positions of the 6-β-side group.

Substitution of the 6-α-position of the penicillin nucleus generally will result in total loss of antibacterial activity. The anomalous temocillin, a 6-α-methoxy-penicillin, however, has a wide range of activity against Enterobacteria, but exhibits a very low level Gram-positive activity [70, 71].

THE CEPHALOSPORINS & CEPHAMYCINS.

Like the penicillins, the cephalosporins usually have a low level of toxicity, and cross-allergenicity is sufficiently low (about 10%) to allow their use in penicillin-sensitive patients [72]. Natural cephalosporins, for example cephalosporin C [36], exhibit a better ratio of Gram-negative/Gram-positive activity than the penicillins, but the intrinsic antibacterial activities of the former are generally lower than the corresponding penicillins. Differences in activity, independent of permeability and β-lactamases, can be correlated to PBP binding characteristics [55]. Although the cephalosporins are resistant to the Gram-positive penicillinases, there are a profusion of Gram-negative β-lactamases that degrade the cephalosporins. The major objectives of research have, therefore, been to increase the intrinsic antibacterial action and broaden the spectrum of activity, partly by improving β-lactamase stability, and pharmacokinetic properties.

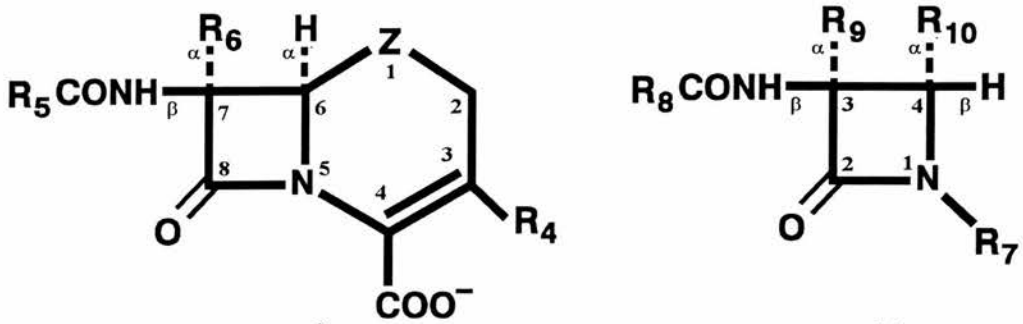


Figure 1.6: Generic structure of the β-lactam antimicrobial agents II: cephems, cephamycins, & non-classic β-lactams

Numbering of ring atoms accords with that of 7-ACA, except monocyclic β-lactams which are numbered clockwise from the lactam bond nitrogen (bottom left). a) Cephems (Z = S, R_6 = H), cephamycins (Z = S, R_6 = OCH_3), oxacephems (Z = O, R_6 = H, OCH_3) b) Monocyclic β-lactams (C-3 side-groups and the N-1→R_7 bond are sterically equivalent to the C-6(7) side-groups and C-3(4)→N-4(5) bond of bicyclic β-lactams respectively): monobactams, sulfazecins, nocardicins.

The cephalosporins [20] (Figure 1.6a: Z = S, R_6 = H), have fewer constraints on the structural modifications that improve biological performance than the penicillins [37, 39]. Careful manipulation of the 3-substituent (R_4), the 7-β-acylamino group (R_5), and substitution of the 7-α-position (R_6) can increase β-lactamase stability and retain useful biological activity. The oxacephalosporin, moxalactam, shows that replacement of the sulphur atom of the dihydrothiazine ring by an oxygen (Figure 1.6a: Z = O) leads to an improvement in antimicrobial activity, whereas this exchange in the 5-membered thiazolidine ring is detrimental to activity. While the opportunity for the modification of cephalosporin nucleus is broader, there are some constraints, for example the use of esters as pro-drugs has not been as successful with the cephalosporins as with the penicillins. The nature of the 7-β-acyl group mainly determines the level and degree of antibacterial activity; the effect on pharmacokinetic properties is less substantial, with the exception of oral absorption, achieved by incorporation of a 7-β-arylglycl group. Changes at the 3-position have occasionally resulted in broadening of the spectrum of activity, but modifications at this position have more influence on the level of activity and

pharmacokinetic properties. Variation of the third important locus of substituent modification, the 7-α-position, is extremely limited, and constrained to a hydrogen ($R_6 = H$ = cephalosporin) or a methoxy group ($R_6 = OCH_3$ = cephamycin); the latter increases resistance to β-lactamases by steric hindrance

The first-generation cephalosporins are unstable in the presence of Gram-negative β-lactamases, and those with a 3'-acetoxy group (Figure 1.6a: $R_4 = CH_2COOH$) are susceptible to metabolic hydrolysis. The general rules governing activity of the penicillins to some extent apply to the cephalosporins, most notably the reduction of Gram-negative activity with the introduction of hydrophobic groups to the side-chain, and the positive effect of some α-substituents (OH, NH_2) on Gram-negative activity. The 3'-acetoxymethyl grouping (Figure 1.6a: $R_4 = CH_2OCOCH_3$), which is present in some the β-lactamase-sensitive cephalosporins, and the 3GC, cefotaxime, is subject to metabolic hydrolysis to give the corresponding 3'-alcohol, with significantly lower antibacterial activity of that of the parent. Whilst detrimental to the earlier cephalosporins' activity and half-life, the result is different with cefotaxime; the activity of the deacetyl derivative of cefotaxime is reduced against the *Enterobacteriaceae*, but still compares favourably to that of other cephalosporins. In addition to avoiding metabolic hydrolysis, modification of the 3-substituent can increase the intrinsic antibacterial activity and improve the pharmacokinetic properties of the cephalosporin. In general, esterification of the 3-substituent has been unprofitable, but one exception is the carbamoyloxy side-group (Figure 1.6a: $R_4 = CH_2OCONH_2$), which occurs naturally in some cephamycins (Figure 1.6a: $R_6 = OCH_3$), has been retained in cefoxitin and incorporated into cefuroxime to help prevent metabolic hydrolysis.

The inclusion of nitrogen nucleophiles, for example pyridines, at the 3-position can increase metabolic stability, and the substitution of sulphur nucleophiles at the same position will provide similar changes in properties. Differences in the intrinsic activities of the penicillins and cephalosporins are attributed to the relative strain of the β-lactam ring, to give different levels of chemical reactivity [55]. Chemical reactivity can be optimised by the choice of side-chain substituents and the rate of ring opening is directly proportional to activity of the cephalosporins against Gram-negative bacteria [73]. Good oral absorption

of cephalosporins, an unusual characteristic of these compounds considering the increased acid stability compared to the penicillins, requires an α-amino substituent on the 7-β-acyl side-chain and small uncharged groups at the 3-position. As observed with the penicillins, resistance to β-lactamase hydrolysis can be improved by steric hindrance around the 7-β-amide group by manipulation of the α-carbon substituents. Incorporation of an α-oxyimino group into the 7-β-acylamino side-chain is a particularly effective method of conferring high level β-lactamase resistance without reduction in antibacterial activity. At this position methoxyimino groups are pharmacokinetically superior to hydroxyimino derivatives. The substitution of a methoxy (or carboxy) group at the 6(7)-α-position [74], as seen in the cephamycins, protects the molecule more efficiently from β-lactamase degradation than any 6(7)-β-position modification. It is interesting to note that 7-α-methoxy groups only protect the oxacephalosporins (Z = O) from penicillinase activity.

NON-CLASSIC β-LACTAMS.

The stereochemistry of the non-classic β-lactams has been shown to conform to the constraints required for activity observed in the penicillins, cephalosporins, and cephamycins. The increased reactivity of the ring system in the penems (Figure 1.5c page 18: X = CH_2 and the conjugated C-2→C-3 double bond) results in the contribution of 6-substituents (R_1 & R_2) being reversed, i.e. the 6-unsubstituted penems and carbapenems are more active than the 6-β-acylamino derivatives. The potent antibacterial activity of the penems is attributed to the ring strain and the electronic effects of the double bond. Monocyclic β-lactam stereochemical configurations of the 3-β-acylamino group and the carboxyl group (Figure 1.6b page 21: R_7) are the same, or similar to, the corresponding positions of the penams and cephems. Changes in activity of various side-chain substituents broadly follow the same trends as seen in the classic β-lactams (e.g. 3-α-methoxy substitutions enhance β-lactamase resistance). The introduction of an α-oxyimino-2-aminothiazol-4-ylacetyl group at the 3-β-position (R_8) into the monobactam structure (Figure 1.6b: R_9 = H or OCH_3, R_{10} = H), will confer high levels of activity against Gram-negative bacteria, as observed in the 3GCs where this type of group has been substituted at the 7-β-position [75, 76].

β-LACTAMASE INHIBITORS.

Apart from the incorporation of structural features in the β-lactam molecule to help prevent inactivation by β-lactamases, specific β-lactamase inhibitors can also be combined with a β-lactam for therapeutic use. Compounds, without a β-lactam ring, exist that are specific or non-specific inhibitors of β-lactamases, however, the most successful candidates for clinical use are based on a β-lactam fused-ring structure. Of the β-lactam containing inhibitors two groups exist [77]:

- Penicillins and cephalosporins that bind, usually reversibly, to the β-lactamase and are hydrolysed, by the normal enzymatic process, to respective penicilloic acid and cephalosporic acids at a very slow rate. β-Lactams in this class are generally competitive inhibitors with a high affinity for the enzyme (low K_m) but with a low V_{max}.
- β-lactam compounds, named 'suicide' inhibitors, bind to the β-lactamase active-site and during the course of a normal hydrolytic action undergo a secondary reaction to stabilise the enzyme-substrate complex. This results in irreversible inhibition of the β-lactamase.

Compounds in the former group require high concentrations to afford protection to the 'antimicrobial' β-lactam with which it is being co-administered. As a consequence the biological activity of this particular combination is variable. Inhibition depends on competitive inhibitor-enzyme binding, but the effect can be transient as a result of the slight hydrolytic susceptibility of the inhibitor.

The second group of agents, also known as 'progressive' β-lactamase inhibitors, can exhibit synergistic antibacterial properties when combined with 'antimicrobial' β-lactams. Clavulanic acid (Figure 1.5b page 18: X = O, $R_1 = NH_3$, R_2 & R_3 = H), sulbactam (Figure 1.5a: X = SO_2, R_1, R_2 & R_3 = H), tazobactam (Figure 1.5b: X = SO_2, ,R_1, R_2 & R_3 = H, and a 2-β-triazol-1-ylmethyl group), certain carbapenems and several semi-synthetic β-lactam derivatives are members of this class of inhibitor. These inhibitors all contain a β-lactam ring that undergo enzyme attack to form the normal acyl-enzyme complex, but then undergo hydrolysis of the β-lactam bond at a slow rate. An

elimination reaction that results in the formation of a double-bond between C-5 and C-6 is facilitated during β-lactam-bond hydrolysis by the acidic 6-α-proton and good 6-β-leaving group of these compounds. The unsaturated acyl-enzyme complex is sufficiently stable to prevent elimination of the hydrolysed product from the active site of the enzyme. The most efficient drugs of this type have high affinity for the β-lactamase active-site, but undergo slow hydrolysis and release of the penicilloic acid product. Describing these agents as 'irreversible' inhibitors is not entirely true as partial recovery has been observed under certain conditions [78]. Esterification of this type of inhibitor does not appear to greatly lower the β-lactamase inhibiting properties [77].

7. THE BACTERIAL CELL WALL

The differences that exist between the bacteria and mammalian cells are ideal targets for antimicrobial agents. In theory, chemicals that will inhibit a process that only takes place in bacteria will not affect a mammalian cell. The obvious distinction between bacteria and animal cells is their structure. Ironically, the evolution of a protective cell wall by bacteria has served as a means of its own destruction. A number of effective antimicrobial agents owe their action to their ability to inhibit bacterial cell wall synthesis, a process with no parallel in animal cells. The biosynthesis of peptidoglycan in both Gram-positive and Gram-negative bacteria is essentially the same; not surprising considering both "types" of bacteria are believed to have evolved from a common ancestor [46].

7.1. PEPTIDOGLYCAN SYNTHESIS

The peptidoglycan biosynthesis pathway has been divided into three stages [79] depending on the location within the bacterium of the reactions: cytoplasm, membrane, and the bacterial cell wall (Figure 1.7 page 26).

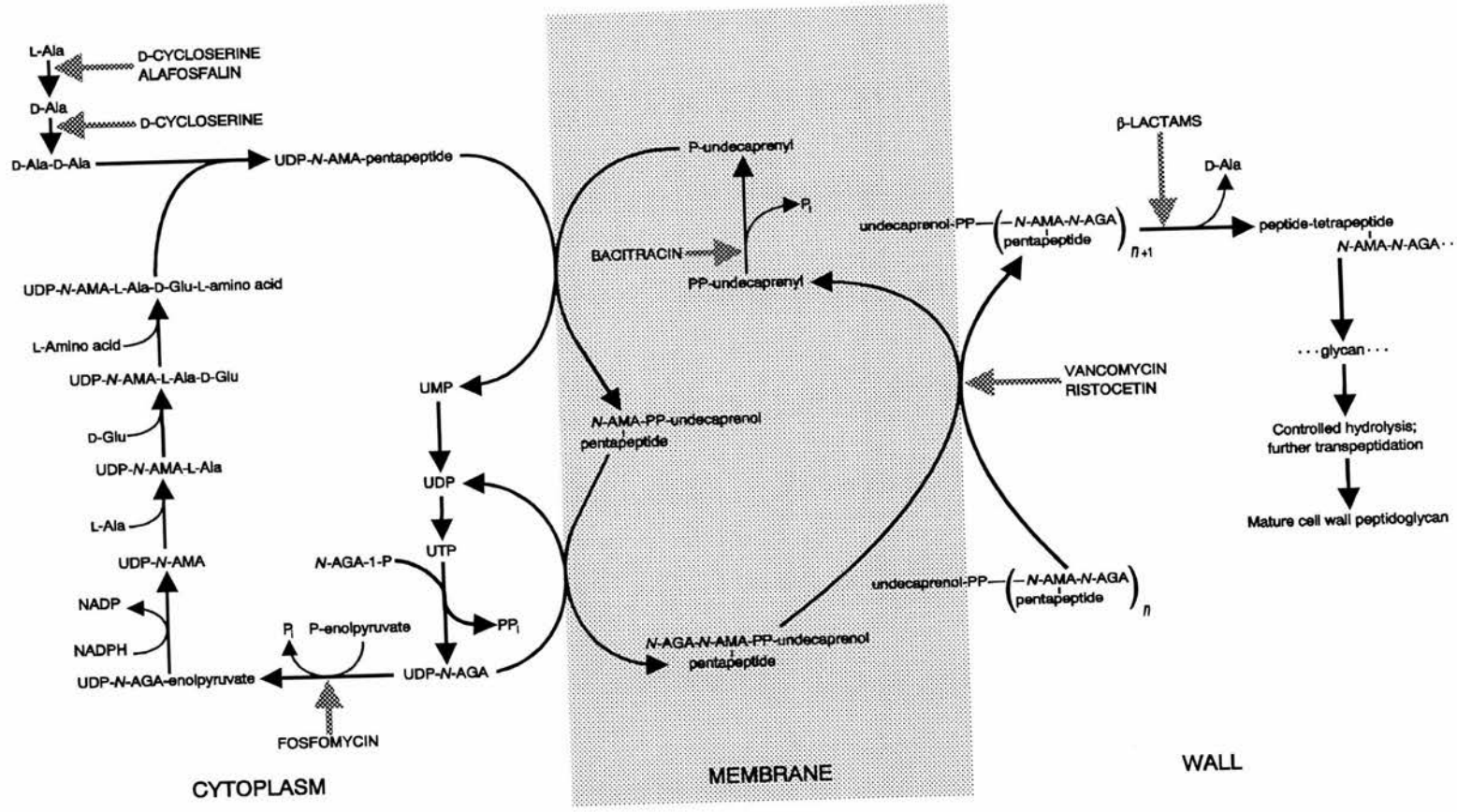


Figure 1.7: Peptidoglycan synthesis & the sites of inhibition by antibiotics.

Points of inhibition by antimicrobial agents are indicated with the shaded arrows (⇒). Abbreviations: D-Ala, D-alanine; L-Ala, L-alanine; L-Ala-D-Ala, L-alanyl-D-alanine; D-Glu, D-glutamic acid; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; UDP-*N*-AGA, uridine-diphosphate-*N*-acetylglucosamine; UDP-*N*-AMA, uridine-diphosphate-*N*-acetylmuramine. After Reynolds (1985) [79].

7.2. INHIBITORS OF PEPTIDOGLYCAN SYNTHESIS

Inhibitors of peptidoglycan anabolism will not have any deleterious effect on an eukaryotic host, except for the undecaprenyl-phosphate inhibitors which may interfere with glycoprotein or sterol biosynthesis. Peptidoglycan synthesis inhibitors may be categorised into three groups, each corresponding to the three stages of biosynthesis: cytoplasmic, cytoplasmic membrane, and the cell wall. The earlier the point of inhibition in the pathway, however, the deeper into the cell the inhibitor will have to penetrate.

β-Lactam antibiotics act on the final stage of the peptidoglycan biosynthetic pathway. The site of this reaction, the inside of the outer cell membrane, requires that these antimicrobial agents only have to penetrate a single membrane, often by passive diffusion through porins. β-Lactams act on the transpeptidation reaction that cross-links the peptidoglycan after transglycosylation. These antibiotics have been shown to be analogues of the D-alanyl-D-alanine terminus of the *N*-AMA-*N*-AGA-pentapeptide [80] (Figure 1.8), a normal substrate of some PBPs.

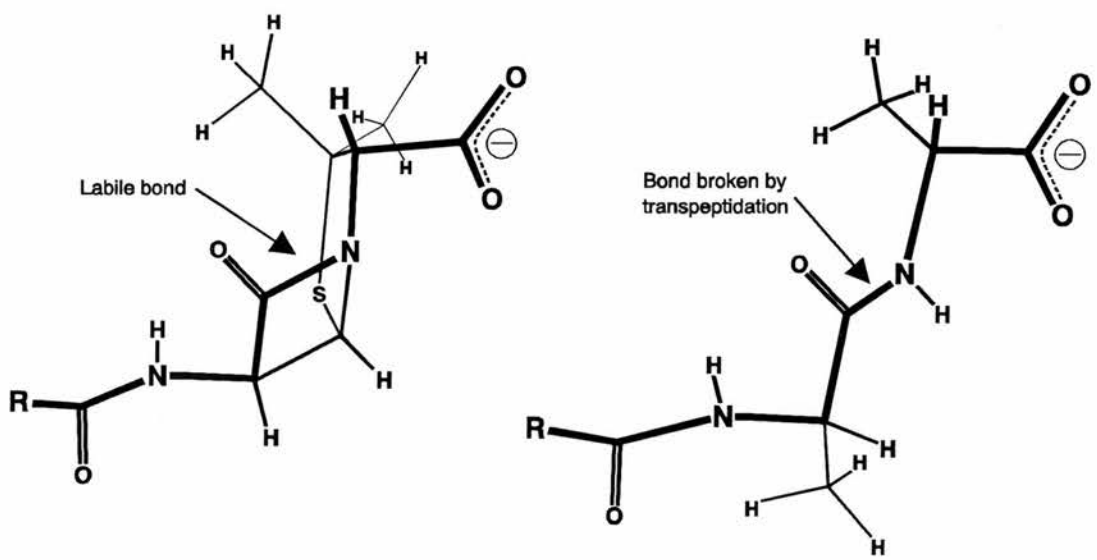


Figure 1.8: Structural comparison of a penicillin with D-alanyl-D-alanine

Chemical structures of a penicillin (left) and the D-alanyl-D-alanine end group of the peptidoglycan precursor (*N*-AGA-*N*-AMA-pentapetide) (right). After Strominger *et al* (1967) [80]

Gram-positive bacteria have about four or five PBPs but Gram-negative organisms have about six main types, and different β-lactam agents have varying affinities for each PBP which can be correlated with the morphological effects observed through PBP inhibition. The properties of the various PBPs present in *E. coli* have been elucidated [81] (Table 1.4). Access to the PBPs of Gram-positive bacteria is not a problem. Penetration through the outer cell wall of Gram-negative bacteria, however, could be a problem for small polar (hydrophilic) molecules if it were not for the OMPs that form water filled channels called porins.

Porin channels in the *Enterobacteriaceae* do not restrict penetration of these antibiotics through the outer membrane to any significant extent [83], but differences in the rate of penetration can explain some of the differences in MICs of β-lactam antibiotics. The penetration rate of a drug through the outer membrane is also important when β-lactamases are present in the bacterium. A slow rate of diffusion into the cell, through loss or mutation of certain porins or poor drug design, may result in a resistant phenotype in the presence of a β-lactamase, when the enzyme alone may not be sufficient to confer resistance on the host. Mutation of the active-site of the PBPs may affect binding of the peptidoglycan synthesis inhibitors and thus also result in resistance of the β-lactam target.

PBP‡	M. Wt§	Enzyme activity	Function
1A, 1B	91000	Transpeptidase (wall) Transglycosylase	Cell elongation
2	66000	Transpeptidase	Longitudinal growth
3	60000	Transpeptidase (septum) Transglycosylase	Septum formation
4	49000	Carboxypeptidase	Lipoprotein attachment
5	42000	DD-Carboxypeptidase	Limitation of cross-linking
6	40000	DD-Carboxypeptidase	Limitation of cross-linking

Table 1.4: The penicillin-binding proteins of *Escherichia coli*

‡ PBP, penicillin-binding protein. § M.Wt, molecular weight. From Franklin & Snow(1989) [82]

8. THE CLASSIFICATION OF β-LACTAMASES

Abraham & Chain [23] were the first to describe the phenomenon of enzymatic degradation of penicillin by 'penicillinases' in 1940; the same year as penicillin was first purified in a sufficiently stable form in order that it could be used clinically [14, 18]. Abraham & Chain [23] identified the presence of penicillinase in a penicillin-resistant Gram-negative bacilli and in a penicillin-sensitive *Micrococcus* and thus postulated that the presence or absence of β-lactamases was not the sole factor in determining the penicillin-resistance phenotype. Staphylococcal penicillinases were described a few years later [84, 85] which, as a result of their selection to epidemic proportions through penicillin use, have commanded a huge research effort, even to the present day [28]. It rapidly became apparent that β-lactamases from different sources were not the same and exhibited variations in enzymatic properties [86, 87]. The introduction of semi-synthetic penicillins highlighted the variations in substrate profiles, especially between Gram-positive- and Gram-negative-*derived* enzymes [88, 89].

Although transferability of antibiotic-resistance had been noted in the 1940s [24] a majority of the early work on transmissible drug resistance was done in post-war Japan [90]. Epidemic multiple-drug resistant shigellosis in Japan prompted a huge research effort and led to the formulation of an explanation for resistance transfer by two groups of workers simultaneously [91, 92]. Despite some earlier reports in English [93-96], it was not until Watanabe reviewed the topic in 1963 [26] that the scientists in Europe and North America were comprehensively introduced to 'R-factors': extra-chromosomal genetic elements, or plasmids, consisting of DNA that encode resistance determinants. Even then it was not until Datta & Kontamichalou first described penicillinase-mediated penicillin-resistance encoded on R-factors in 1965 [97] that the multiplicity of the β-lactamase phenomenon began to be fully realised.

Once it was apparent that there were many different β-lactamases in the environment classification schemes began to be introduced to attempt to group these enzymes either by biochemical characteristics, by structure, or by amino acid sequence depending on the needs of the classifier.

8.1. THE RICHMOND & SYKES CLASSIFICATION OF β-LACTAMASES

The earliest schemes were, as would be expected, very simple, sub-dividing the enzymes into relatively few groups [98-101]. As the number of characterised penicillinases and cephalosporinases increased the complexity of the classification schemes also increased until Richmond & Sykes produced a more comprehensive strategy in 1973 [102]. The 'Richmond & Sykes classification', as it has become known, is still used today by some workers, especially those studying chromosomally-encoded β-lactamases.

The scheme (outlined in Table 1.5 page 31) initially divided β-lactamases into five classes (designated by roman numerals I to V) depending upon the substrate profile of the enzyme. The first class (I) was divided further into four sub-groups: a, b, c, and d. Neu [103] introduced an extra class (VI) to accommodate the β-lactamases of *Bacteroides* spp. in 1986. The major disadvantages of this classification scheme are that it is entirely based on biochemical characteristics. β-Lactamases that are entirely unrelated, apart from in the substrate profile, are grouped together. It would be reasonable to assume that enzymes may have evolved from different species under the same selective pressures, therefore, the biochemical properties of the enzymes are similar but protein structures are quite distinct

As the techniques for analysing β-lactamases improved and as new techniques became available the criteria for categorising the enzymes could become more stringent. The most significant advance in aids for the study of β-lactamases in the 1970s was the discovery of the chromogenic cephalosporin, nitrocefin [104], followed by the development of the technique of isoelectric focusing (IEF) [105]. This allowed the presence of β-lactamases to be easily ascertained and also the ready determination of isoelectric point (pI) of the enzyme.

Sykes & Matthews [106] modified the classification of β-lactamases derived from Gram-negative bacteria through the use of these techniques and also included the molecular weight of the enzyme. Sykes & Matthew initially divided Gram-negative β-lactamases into chromosomally- or plasmid-mediated enzymes and then sub-divided the former into three groups: a, b, and c (penicillinases, cephalosporinases, and broad-spectrum enzymes). Plasmid-mediated β-lactamases were divided into

two groups (a & b) depending on substrate profile (hydrolysis, or not, of methicillin and isoxazoyl penicillins). A final plasmid-mediated group (c) was added to contain the β-lactamases that do not fit into either group a or b [107].

As the number of plasmid-encoded β-lactamases identified increased the Richmond & Sykes classification became insufficient to deal with the profusion of hydrolytically similar enzymes. The Sykes & Matthew modification compensated slightly for this shortfall, but was not extensive enough. Several classification schemes for both plasmid-mediated and chromosomally-mediated enzymes have been devised separately, but have led to some confusion over nomenclature [108].

8.2. THE AMBLER CLASSIFICATION OF β-LACTAMASES

The description of a classification, based on comparisons of the amino acid sequences of enzymes, by Ambler in 1980 [109], was a major advance in β-lactamase characterisation. Most of the preceding classifications, apart from the

Class		Activity§	Source	Other features
I	a	Cephalosporinase	Chromosomal	Inducible e.g. P99 from *Enterobacter cloacae*
	b			Constitutive e.g. AmpC from *E. coli*
	c			Inducible e.g. cefuroximase & cefotaximase from *Proteus vulgaris*
	d			Inducible e.g. Sabath-Abraham enzyme from *Pseudomonas aeruginosa*
II		Penicillinase	Chromosomal	Rare enzymes but noted in *Proteus mirabilis*
III		Penicillinase & Cephalosporinase	Plasmid	Inhibited by cloxacillin & pCMB e.g. TEM- & SHV-*type* enzymes
IV		Penicillinase & Cephalosporinase	Chromosomal	Resistant to inhibition by cloxacillin but inhibited by pCMB.
V		Penicillinase	Plasmid	Resistant to inhibition by cloxacillin & pCMB e.g. OXA- & PSE-*type* enzymes
VI		Cephalosporinase	Chromosomal	Found in *Bacteroides* spp.

Table 1.5: Richmond & Sykes classification scheme for β-lactamases

§ Activity predominantly, but not necessarily absolutely, penicillinase or cephalosporinase in nature

Richmond & Sykes method [102], are insufficiently broad or detailed to encompass the increasing number of β-lactamases and are no longer used.

Five classes have now been attributed to the Ambler classification (Table 1.6 page 33). Originally two classes (A and B) were described [109] based on the amino acid sequence differences of six β-lactamases: *Bacillus cereus* 569/H type I and type II, *B. licheniformis* 749/C, *Staph. aureus* PC1, TEM-1_{pBR322} and TEM-2_{RP4}. Sequence homology suggested that five of these β-lactamases belonged to the same evolutionary distinct group: active-site serine penicillin-recognising enzymes. The sixth β-lactamase, *B. cereus* 569/H type II, a metalloenzyme, lacked a significant degree of sequence homology. Additional classes C [110], D [111], and E [112, 113] have been added in the last 13 years. It maybe interesting to note that in the literature Bush [114] is often quoted as proposing a modification to the Ambler classification: the paper actually proposes a scheme based on biochemical characteristics, i.e. a Richmond & Sykes style classification.

Enzymes belonging to Ambler classes A, C, and D, as well as the PBPs, are members of a superfamily of active-site serine penicillin-recognising enzymes [115]. β-Lactamases of classes A and D are thought to have evolved from the penicillin-sensitive D-alanyl-D-alanine carboxypeptidases (DD-peptidases) involved in bacterial wall peptidoglycan anabolism [80]. Kelly *et al.* [116] and Samraoui *et al.* [117] compared the primary and tertiary structures of the *Streptomyces* R61 DD-peptidase with the *B. licheniformis* 749/C and *B. cereus* type III β-lactamases respectively. Although the β-lactamases and carboxypeptidases have different biochemical properties (but recognise the same substrates) and primary structures, they show significant similarity in the spatial arrangement of the secondary structure (Figure 1.9 page 34). Until recently only classes A and D contained examples of plasmid-mediated β-lactamases; however class C plasmid-mediated resistance has recently been observed [118, 119].

CLASS A β-LACTAMASES

Class A enzymes differ widely in biochemical and physical properties but preferentially hydrolyse penicillins and contain about 260-270 amino acid residues. These β-lactamases have a serine residue at the active-site, show

considerable sequence homology with the DD-peptidases [116, 117] and include the TEM- and SHV-*type* β-lactamases (acronyms of a patients name, 'Temoneira', and the biochemical characteristic 'Sulphydryl variable' [108]).

Huletsky *et al.* [120] constructed a phylogenetic tree of the class A β-lactamases. The cladogram obtained by the analysis of 18 β-lactamases (Figure 1.10 page 35) showed that class A enzymes divide into Gram-negative and Gram-positive derived branches. The only anomaly in this sub-division was the plasmid-mediated ROB-1 β-lactamase of *H. influenzae*. The close relationship of this Gram-negative species' enzyme to Gram-positive enzymes may be the result of horizontal gene transfer [120, 121]. Considering that gene transfer is entirely possible, especially associated with antibiosis [45], it is surprising that of the 18 β-lactamases studied by Huletsky *et al.* [120] only one is 'misplaced'.

Class	M. Wt	Type	Activity	Source	Examples
A	~29,000	Active-site serine	Broad-spectrum β-Lactamase inhibitor sensitive	Chromosomal	*Bacillus cereus* I
				Plasmid	TEM-*type* SHV-*type*
B	Variable	Metalloenzymes (ZnII)	Inhibited with EDTA. β-Lactamase inhibitor sensitive.	Chromosomal	*Bacillus cereus* II *Bacteroides fragilis*
C	~40,000	Active-site serine	Broad-spectrum β-Lactamase inhibitor resistant	Chromosomal	*E. coli* AmpC
				Plasmid	MIR-1, BIL-1
D	Variable	Active-site serine	Carbenicillinase or oxacillinase	Plasmid	OXA-*type* PSE-*type*
E	~120,000	Metalloenzymes (ZnII)	Inhibited with EDTA β-Lactamase inhibitor resistant	Chromosomal	*Xanthomonas maltophilia* L1

Table 1.6: Summary of the Ambler β-lactamase classification

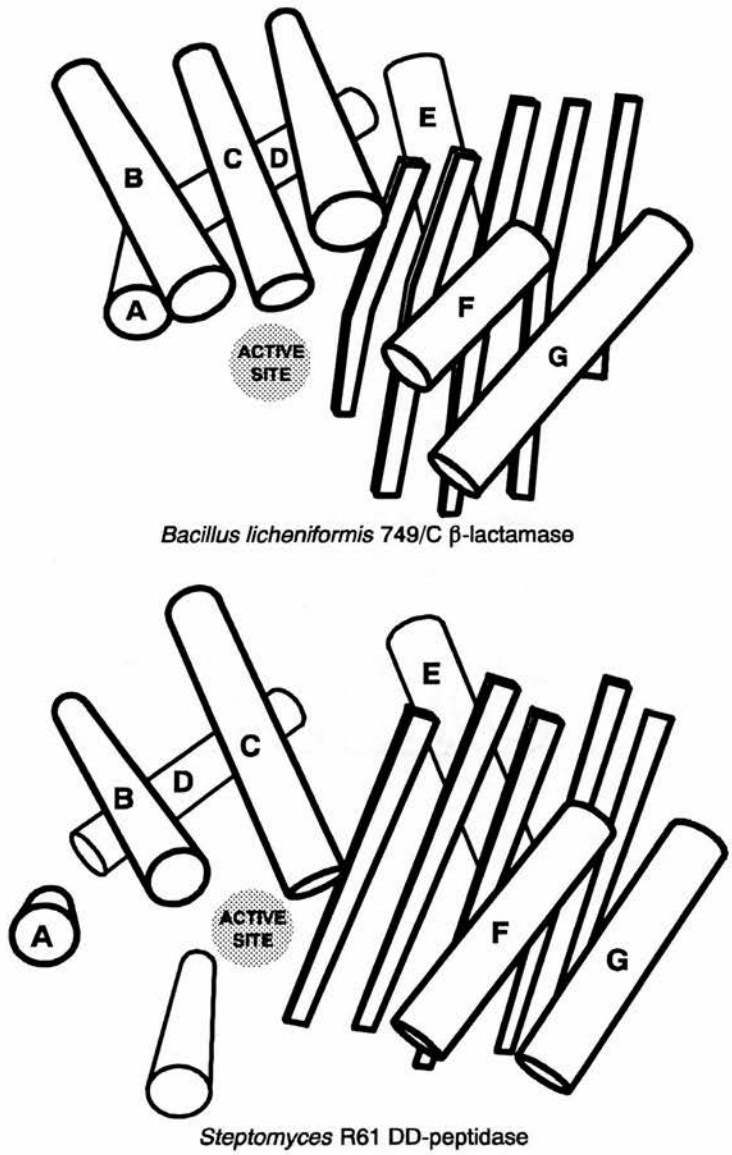


Figure 1.9: Comparison of the tertiary structure of a Class A β-lactamase & a DD-peptidase

Comparable α-helices of enzymes are denoted with the same letters (A to G). Diagram modified from Kelly *et al.*(1986) [116].

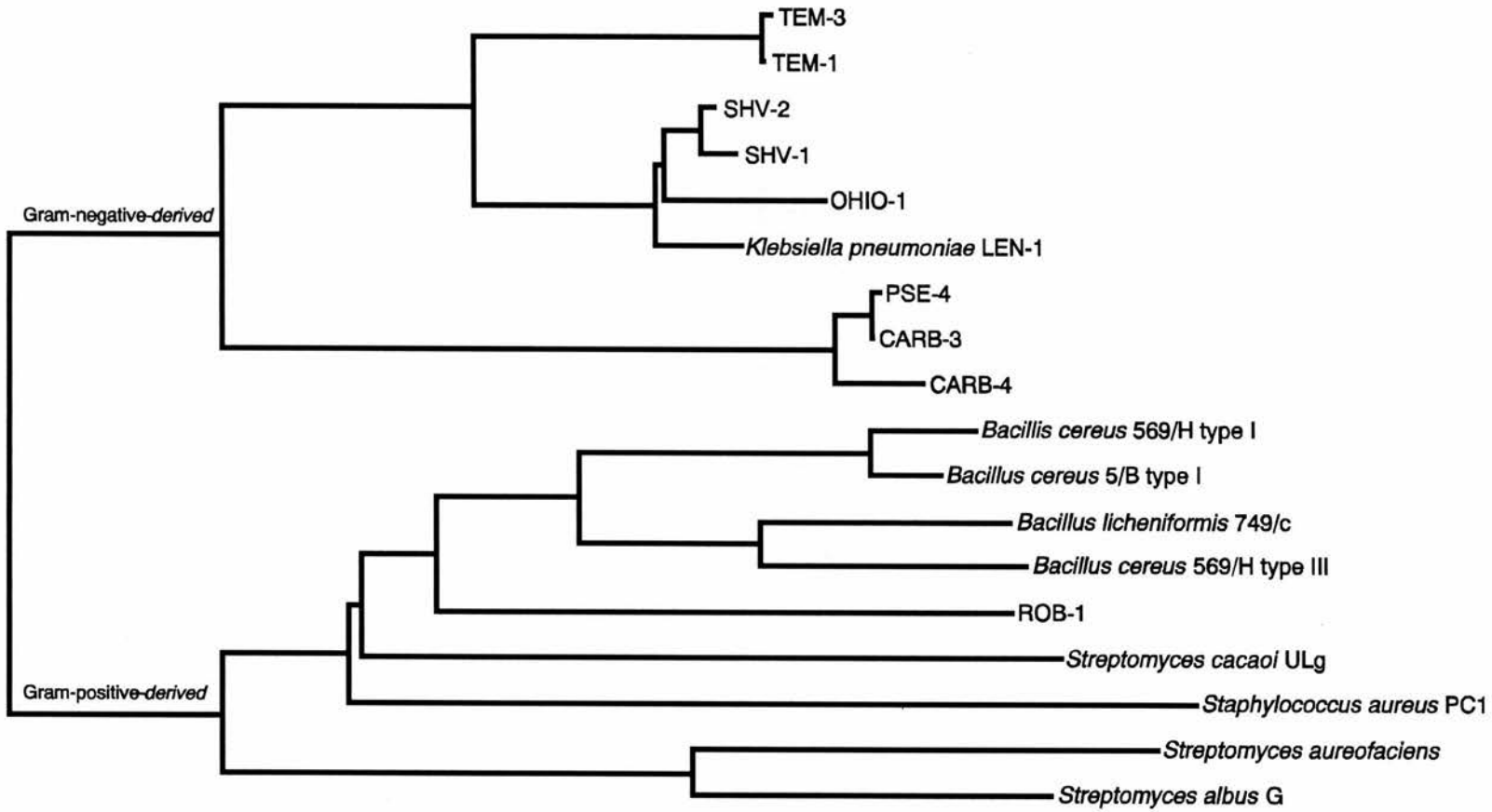


Figure 1.10: Cladogram of 18 Ambler Class A β-lactamases

Branch lengths represent relative phylogenetic distances. The cladogram divides into Gram-positive-*derived* and Gram-negative-*derived* groups on the basis of amino acid sequence alignment. See Huletsky *et al.* (1990) [120] for details.

The Gram-positive-*derived* enzymes are mostly chromosomally mediated (except the plasmid-mediated ROB-1 and *Staph. aureus* PC1 enzymes), which compares with the predominantly plasmid-mediated nature of the Gram-negative-*derived* group (exceptions include the chromosomally mediated *Klebsiella pneumoniae* enzyme LEN-1) [108, 120]. Evolutionary distances between Gram-positive enzymes are typically much longer than those of the Gram-negative enzymes. Huletsky *et al.*[120] noted that despite this there was no fundamental change in substrate profile and concluded, in agreement with Woese [122], that the divergence of genotype was not mediated by an external selective pressure. It was concluded that the Gram-positive enzymes appeared earlier in evolution than the Gram-negative enzymes, but both groups had evolved in parallel from a common ancestor [120].

Comparison of the tertiary structures of the Gram-positive class A enzymes, *Staph. aureus* PC1 [123], *B. licheniformis* 749/C [116, 124], and *B. cereus* type I [117, 125] with that of the Gram-negative plasmid-mediated β-lactamase TEM-1 [126, 127] reveals considerable conservation of the spatial arrangement of secondary elements, lending additional weight to the 'common ancestor' hypothesis. Several amino acids have multiple codons, but serine is unique in having a large set of codons which divide into two groups, TCN and AGY, that cannot be converted to the other via a single nucleotide change. Class A β-lactamases (and class C and D) possess a serine residue at the active-site. This residue, as a result of its role in catalysis, is highly conserved and loss results in a reduction of activity if not total inactivity [128, 129]. Brenner [130] proposed that two evolutionary distinct groups (those with a TCN serine codon and those with an AGY serine codon) of active-site serine proteins have evolved from a common ancestor. He proposed that of the two possible ancestral codons, ACN for threonine and TGY for cysteine, the latter was the most probable on the basis of catalytic function. Replacement of the TEM-1_{pBR322} active-site serine with cysteine reduces, but does not totally abolish activity [128], as observed when the serine is replaced with threonine [129]. Comparison of the active-site serine codons of the class A enzymes analysed by Huletsky *et al.* [120] shows that in general the Gram-negative-*derived* β-lactamases use the AGY

serine-codon and the Gram-positive-*derived* enzymes serine-codons are of the TCN-*type* suggesting dual pathways of descent.

CLASS B β-LACTAMASES

Class B enzymes are metalloenzymes that require a divalent zinc atom bound to two histidines and a cysteine residue at the active site [109]. Class B enzymes are generally slightly smaller than class A enzymes and lack sequence homology (either DNA or amino acid) suggesting the two classes had independent origins.

CLASS C β-LACTAMASES

Only classes A and B were proposed by Ambler in the original classification of the β-lactamases he analysed. He did, however, foresee the expansion of the scheme to include enzymes that did not fit into the original two groups [109]. Jaurin & Grundström [110] were the first to modify the Ambler classification after analysing the chromosomal ampC β-lactamase of *E. coli*. Class C enzymes lack sequence identity with either class A or class B enzymes. Like class A enzymes, class C β-lactamases are active-site serine enzymes but they are much larger than the preceding two classes, and preferentially cephalosporinases [110, 131, 132] even to the extent of conferring resistance to 3GCs and β-lactamase inhibitors [118, 119].

CLASS D β-LACTAMASES

As the DNA and amino acid sequences of an increasing number of β-lactamases were determined it became apparent that another group needed to be added to the Ambler classification. PSE-2 β-lactamase [133] was found to have significant similarity to the OXA-1 and OXA-2 β-lactamases [111, 134], all of which were sufficiently different from class A, B, and C, for Ouellette *et al.* [111] to propose a new class. Class D was designated to include the plasmid-mediated PSE- and OXA-*type* enzymes (acronyms of '<u>Pse</u>udomonas-specific' and '<u>oxa</u>cillin-hydrolysing' respectively [108]) and other enzymes that exhibit carbenicillinase or oxacillinase activities. The PSE-4 β-lactamase, although exhibiting the same characteristics of other PSE-*type* enzymes, has been allocated to class A on a basis of DNA sequence homology [135].

CLASS E β-LACTAMASES

Class E is the most recent addition to the Ambler classification. The L1 β-lactamase of *Xanthomonas maltophilia* [112, 136] is a metalloenzyme requiring a divalent zinc ion at the active-site for activity, as in class B. The differences in biochemical properties and lack of sequence homology with class B enzymes has prompted the suggestion by Sanders [113] that this fifth class be created to accommodate this unique β-lactamase.

8.3. THE BUSH CLASSIFICATION OF β-LACTAMASES

After Bush & Sykes [137] detailed guidelines for the study of the biochemical characteristics of β-lactamases a classification with a more sensitive analysis of enzyme kinetic parameters needed to be outlined. A modification of the biochemical classification of β-lactamases [100, 102, 106] was proposed by Bush in 1988 [114]. This 'Bush classification' catalogued a large number of β-lactamases in great detail [138-140] (Table 1.7 page 40). An initial review [114] defined three classes (1, 2, and 3) in which class 2 was further divided into five sub-groups (annotated a to e). The more detailed description [138-140] expanded the scheme to include two extra classes: class 2b' and class 4. The classification tried to take account of molecular aspects as well as biochemical characteristics, but was not particularly successful in this respect.

CLASS 1 (CEP-N) β-LACTAMASES

CEP-N (Cephalosporinase not inhibited by clavulanic acid) are preferentially cephalosporinases and are not inhibited by 10μM clavulanate or ethylenediaminetetraacetate (EDTA). Representative enzymes of this class are the chromosomal β-lactamases of Gram-negative bacteria, for example the *E. coli* ampC enzyme, or the Sabath & Abraham enzyme of *Ps. aeruginosa* and *Serratia marcescens*. This group does not exclude plasmid-mediated β-lactamases, for example CEP-1_{R22K} from *Proteus mirabilis*.

CLASS 2 β-LACTAMASES

This large group, divided into six sub-classes, consists of a variety of β-lactamases all inhibited by clavulanic acid (hence the 'Y' suffix of each sub-title). Class 2a (PEN-Y) enzymes are classical penicillinases characteristically of

Gram-positive origin. Although predominantly chromosomally encoded a few notable exceptions are plasmid-mediated, for example the PC1 β-lactamase of *Staph. aureus*. Class 2b (BDS-Y), broad-spectrum enzymes with both cephalosporinase and penicillinase activity, are predominantly plasmid-mediated and contain the ubiquitous TEM-1 and SHV-1 β-lactamases. These enzymes, however, do not exhibit any ESBL activity. The later designation of class 2b' (EBS-Y) was added to the Bush classification to accommodate the novel extended-spectrum enzymes, e.g. TEM-3, TEM-5, SHV-5, which readily hydrolyse the 3GCs. Classes 2c (CAR-Y) and 2d (CLX-Y) include penicillinases that also exhibit carbenicillinase and oxacillinase activity respectively. Examples of these classes, again predominantly plasmid-meditated, include some of the PSE-*type* enzymes, CARB-*type* enzymes and the OXA-*type* enzymes. The final class 2 group, 'e' (CEP-Y), are cephalosporinases that uniquely exhibit immunological properties similar to those of the penicillinases.

CLASS 3 (MET-N) β-LACTAMASES

Class 3 enzymes are all metalloenzymes, usually requiring a ZnII ion at the active-site for activity. Since the criteria for designation into this class is being a metallo-β-lactamase the hydrolytic properties are distinctly varied; however, none are inhibited by clavulanic acid, and as would be expected all are inhibited in the presence of the chelating agent EDTA. This group includes the *Xanthomonas maltophilia* L1 and the *B. cereus* type II metalloenzymes that have been separated under the Ambler classification (see Section 8.2 pages 31-38).

CLASS 4 (PEN-N) β-LACTAMASES

The PEN-N class has been added to the original Bush classification to include those enzymes that are predominantly penicillinase in activity but are not inhibited by clavulanic acid. The inhibition characteristics of EDTA on this group is variable because of the 'any other β-lactamase' nature of this class.

Class	Sub-title	Substrates	Inhibited by:		M. Wt. (10^3)	Examples:
			Clav	EDTA		
1	CEP-N	Cephalosporins	-	-	30 → 40	Gram-negative chromosomal enzymes
2 a	PEN-Y	Penicillins	+	-	15 → 34	Gram-positive penicillinases
b	BDS-Y	Penicillins, cephalosporins	+	-	17 → 29	TEM-1, TEM-2, SHV-1
b'	EBS-Y	Penicillins, cephalosporins, incl. 3GCs	+	-	20 → 29	TEM-3, TEM-5 etc.; SHV-2, SHV-4 etc.
c	CAR-Y	Penicillins, carbenicillin	+	-	12 → 34	PSE-1, PSE-3, PSE-4
d	CLX-Y	Penicillins, cloxacillin	+	-	23 → 43	OXA-1, PSE-2
e	CEP-Y	Cephalosporins	+	-	26 → 48	*Proteus vulgaris* FEC-1 *Xanthomonas maltophilia* L2
3	MET-N	Variable	-	+	22 → 120	*Bacillus cereus* type II, *Xanthomonas maltophilia* L1
4	PEN-N	Penicillins	-	Variable	26 → 85	*Pseudomonas aeruginosa* LCR-1

Table 1.7: Summary of the Bush classification of β-lactamases

Abbreviations: BDS- prefix, broad spectrum β-lactamase; CAR- prefix, carbenicillin hydrolysing; CEP- prefix, cephalosporinase; Clav, 10μM clavulanic acid; CLX- prefix, cloxacillin hydrolysing; EBS- prefix, extended spectrum β-lactamase; MET- prefix, metallo-β-lactamase; M. Wt., molecular weight; -N suffix, not inhibited by clavulanic acid; PEN- prefix, penicillinase; -Y suffix, inhibited by clavulanic acid. After Bush (1989) [138]

8.4. The Richmond & Sykes, Ambler, and Bush classifications: A critique

Although each of the three of the above classifications, presented chronologically, has its advantages each also has specific disadvantages. The Bush classification, despite being originally proposed as a modification to the Richmond & Sykes scheme, is sufficiently different to be considered as a classification scheme in its own right.

Both the Richmond & Sykes and the Bush methods essentially use the biochemical characteristics of the enzymes studied to classify them. The latter of these two classifications, primarily as a result of the improvements in scientific techniques in the 15 years between the two descriptions, requires a much more detailed examination of the kinetics of a particular enzyme for categorisation than the former. The Ambler system primarily relies on sequence homology to define the classification but does, to some extent, also rely on the identification of biochemical properties. To categorise a particular enzyme by the Ambler scheme requires the DNA or amino acid sequence to be determined. This is not the problem it was when Ambler introduced the concept of this categorisation in 1980 [109]. Advances in molecular biology techniques have allowed the rapid and efficient determination of DNA sequences on a routine basis. The Bush classification makes some attempt to include the genetic details of the enzymes catalogued [138-140], but as can be seen from the inclusion of molecular weights of enzymes (Table 1.7 page 40), each class contains enzymes of very varied size, hence varied ancestry. This adds further weight to the suggestion that many β-lactamases have undergone convergent evolution under the selection of a common antibiotic. Correlation of classification, however, under the Richmond & Sykes and Bush schemes with the evolutionary categorisation of Ambler system is poor.

It is probably the Ambler classification that will ultimately become the standard method of classification of β-lactamases, but as discussed below, this will be supplemented by other systems within each class (A, B, C, D, and E). The determination of DNA sequence is the ultimate method of defining the novelty of a particular β-lactamase [141].

9. TEM- & SHV-*DERIVED* β-LACTAMASES

The most prevalent and successful β-lactamase genes are carried by plasmids which, unlike their chromosomally-encoded counterparts, are usually produced constitutively so that any change in the ability to confer resistance stems from alterations in the structure of the molecule rather than in the regulation of gene expression. The most successful plasmid-encoded β-lactamases are the members of the TEM- and SHV-families [2]. Expression of the TEM-1 β-lactamase is the most common mechanism of resistance to the β-lactam group of drugs in Gram-negative bacteria. This successful resistance gene has most regularly been identified in *Enterobacteriaceae* but is not confined to it. The gene is usually located on the promiscuous transposon Tn*3*, and a series of transposition and rearrangement events have allowed the TEM-1 gene's migration into many bacterial species [107] including *Haemophilus* [142], *Neisseria* [143] and *Vibrio* spp. [144]. The SHV family of β-lactamases, on the other hand, appear to have been derived in *Klebsiella*. The gene of the prototype SHV-1 shares about 65% identity with the TEM-1 gene [120]. Both are classified as Ambler Class A β-lactamases [109] in which the basic shape of the enzyme molecule is conserved [116] and TEM-1 and SHV-1 have very similar biochemical properties.

It is not obvious why TEM-1 should be so prevalent, accounting for about 80% of all plasmid-encoded β-lactamases in clinical *Enterobacteriaceae*, whereas SHV-1 accounts for little more than 1% [3, 145-148]. The transposon, on which the TEM-1 gene resides, may be far more mobile than the DNA that surrounds the SHV-1 gene but this may not be the explanation. The gene of the β-lactamase TEM-2, a very close relative of TEM-1 which differs from it only by a substitution of a single amino acid (glutamic acid to lysine) at position 39 [149, 150], is also located on a transposon. The TEM-2 enzyme never accounts for more than 10% of all plasmid-encoded β-lactamases. There are probably subtle structural differences between the TEM-1 enzyme and either TEM-2 or SHV-1, which produce changes in the biochemical function of the active site of the enzyme that even modern assay techniques are too crude to distinguish. It seems that the active site of the TEM-1 β-lactamase is exquisitely suited to the binding and hydrolysis of the most common penicillins in clinical use.

The earliest clinical strain known to possess an extended-spectrum β-lactamase was isolated in England in 1982 [151]. A strain of *Klebsiella oxytoca* was responsible for an outbreak of infection in a neonatal unit in Liverpool. This strain was originally gentamicin resistant but ceftazidime sensitive and it produced the TEM-1 β-lactamase. Ceftazidime was used to control it, but subsequent isolates of *Kleb. oxytoca* from this unit showed that the strain had become ceftazidime resistant. The ceftazidime resistance gene was transferable and carried on a 141-kb plasmid [151]. Subsequent detailed biochemical analysis showed that the ceftazidime resistance was mediated by a new β-lactamase which hydrolysed ceftazidime with low, but significant, efficiency [151].

Despite the fact that the earliest transferable extended-spectrum β-lactamase has now been shown to have originated in an English strain, it is on the mainland of Europe where most of these enzymes have been found and where they were first reported [152, 153]. In 1983, three strains of *Kleb. pneumoniae* and one *Serratia marcescens* isolated in West Germany were demonstrated to confer transferable resistance to cefotaxime [154]. The plasmid mediated β-lactamase was a modification of the Klebsiella enzyme SHV-1 and was designated SHV-2 [155]. The nucleotide sequence of SHV-2 showed a single nucleotide point mutation changing the amino acid at position 238 from a glycine to a serine [120]. These observations spawned a series of reports of transferable plasmid-encoded resistance to 3GCs and almost all these broad spectrum enzymes have been found to be modifications of either the TEM or SHV groups of β-lactamases [156]. The extended-spectrum SHV- and TEM-*derived* enzymes have been identified in clinical isolates on every continent [108, 152]. These enzymes have been found in many *Enterobacteriaceae*, but predominantly in *Klebsiella*, and are often encoded on large transferable plasmids. These could have arisen either from direct selection, or by *in vivo* conjugation.

9.1. MUTATIONS OF THE TEM- AND SHV-β-LACTAMASES

From the first example of cephalosporin selection of TEM-*derived* mutants in Liverpool [151], the extended-spectrum β-lactamases seem to have emerged spontaneously as a response by clinical isolates to the selective pressure of the 7-oxyimino cephalosporins. Many of the extended spectrum β-lactamases have now been sequenced, or the mutations have been deduced after oligonucleotide

probing ('oligotyping') [157], and relatively few point mutations have occurred in the genes of the TEM- and SHV-type enzymes (Table 1.8 and Table 1.9 page 45).

β-Lactamases defined as Ambler Class A have differed in the numbering of amino acid residues depending on whether a 'natural' or 'sequential' method is used. The TEM- and SHV-enzymes have generally been numbered, at the level of amino acid sequence by one of two methods. Sutcliffe's numbering system [150] is derived from the natural sequence, after the determination of the sequence of TEM-1_{pBR322}, and places the active-site serine at residue number 68. The second method used regularly is based upon the consensus sequence obtained by Ambler [109] when his β-lactamase classification was first proposed. This latter system places the active-site serine at residue number 70. The Sutcliffe system generally numbers residues at two less than the Ambler system, but numbers vary towards the C-terminal end of sequences because of deletions. For example, in TEM-1, residue 261 under the Sutcliffe numbering system is equivalent to residue 265 of the Ambler system. As a result of the confusion these dual methods were causing, Ambler *et al.* [158] proposed that the standard numbering system places the active-site serine at residue 70 and that standard numbers be indicated by the 'ABL' prefix (Class A Beta-Lactamase). The Ambler standard numbering system [158] is used throughout this thesis, but the 'ABL' prefix is not used.

Enzymes	Amino acid at position†		
	205	238	240
SHV-1	Arg	Gly	Glu
SHV-2		Ser	
SHV-3	Leu	Ser	
SHV-4	Leu	Ser	Lys
SHV-5		Ser	Lys
SHV-8			Lys

Table 1.8: Molecular basis of the SHV-*derived* β-lactamases

† Amino acid numbering according to Ambler *et al.* (1991) [158]. Abbreviations: Arg, arginine; Glu, glutamic acid; Gly, glycine; Leu, leucine; Lys, lysine; Ser, serine.

Enzyme	Amino acid at position†						
	39	104	164	237	238	240	265
TEM-1	Gln	Glu	Arg	Ala	Gly	Glu	Thr
TEM-2	Lys						
TEM-3	Lys	Lys			Ser		
TEM-4		Lys			Ser		Met
TEM-5			Ser	Thr		Lys	
TEM-6		Lys	His				
TEM-7	Lys		Ser				
TEM-8	Lys	Lys	Ser		Ser		
TEM-9		Lys	Ser				Met
TEM-10			Ser			Lys	
TEM-11	Lys		His				
TEM-12			Ser				
TEM-13	Lys						Met
TEM-14	Lys	Lys			Ser		Met
TEM-15		Lys			Ser		
TEM-16	Lys	Lys	His				
TEM-17		Lys					
TEM-18	Lys	Lys					
TEM-19					Ser		
TEM-24	Lys	Lys	Ser	Thr		Lys	
TEM-26		Lys	Ser				

Table 1.9: The molecular basis of the TEM-*derived* extended-spectrum β-lactamases

† Amino acid numbering according to Ambler *et al.* (1991) [158]. Abbreviations: Ala, alanine; Arg, arginine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Lys, lysine; Met, methionine; Ser, serine; Thr, threonine.

The point mutations of the SHV- and TEM-*derived* β-lactamases are clustered in five areas of the enzyme (Figure 1.11 page 47), each adjacent to one of seven evolutionary conserved boxes described by Joris *et al.* [115]; area 1 is at position 104 (adjacent to the α-3 helix), area 2 is at position 164 (adjacent to the α-7 helix), area 3 is at position 205 (on the α-9 helix), area 4 is at positions 237-240 (on the β-3 strand) and area 5 is at position 265 (on the β-5 strand). Each of these areas are located in close proximity to the active-site cavity (Figure 1.11) and are thought to open up novel enzyme-substrate interactions involving, in particular the oxyimino substituents of 3GCs [159].

9.2. The Payne & Amyes classification of extended-spectrum β-lactamases

It is current practice to classify β-lactamases by their nucleotide sequence and for most enzymes this works well; however, all the extended-spectrum β-lactamases fall into class A [109] and encompass enzymes whose genes share as little as 35% identity but retain the same overall shape. The lack of correlation between sequence and enzyme function is epitomised with the prototype enzymes TEM-1 and SHV-1; these enzymes are impossible to distinguish biochemically. On the other hand, the gene of TEM-5 has only three nucleotide changes from that of TEM-1 [160] (i.e. > 99.5% identity). The biochemical difference of the resultant TEM-5 β-lactamase and the resistances that it confers are of immense clinical importance. So a classification scheme based entirely on the structure is unhelpful.

A categorisation scheme devised by Payne & Amyes [152], for the plasmid-mediated ESBL, based on the biochemical properties and the resistances that the enzymes confer, shows that the mutations produce three major changes in function. A summary of the original classification is given in Table 1.10 (page 48). Most of the enzymes included in the original classification [152] are members of Ambler class A, however, some have subsequently been found to be members of other classes (e.g. BIL-1 and MIR-1 have now been allocated to Ambler class C).

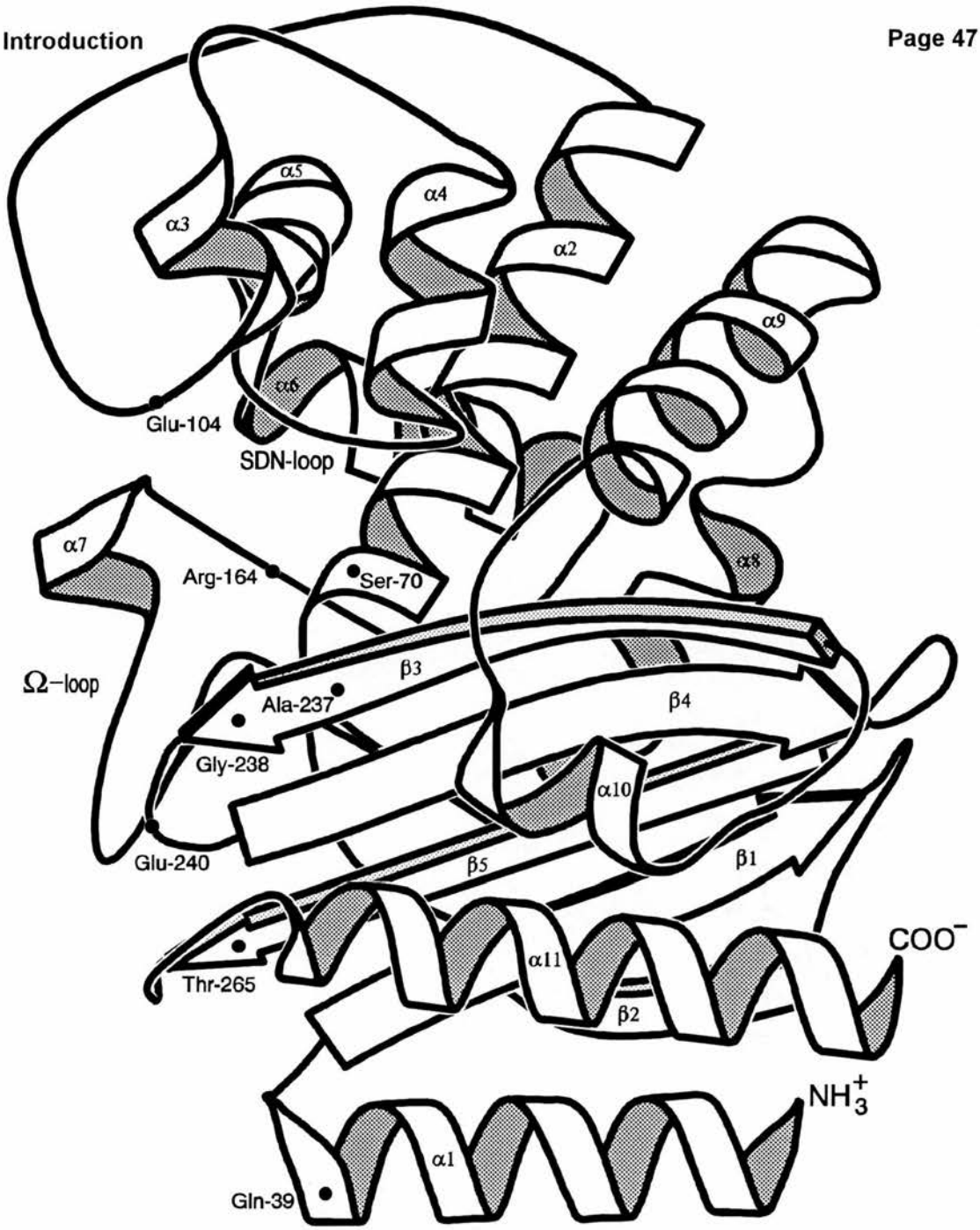


Figure 1.11: Ribbon diagram of the TEM-1 β-lactamase

Numbering of amino acid residues according to the recommendations of Ambler *et al.* (1991) [158]. Diagram is based on the crystal structure of the TEM-1 β-lactamase described by Jelsch *et al.* (1992) [126] and Jelsch *et al.* (1993) [127]. The active site is centred around the serine-70 and bounded by the α3 and α7 helices, β3 sheet, the SDN loop (named after the conserved Serine-Aspartic acid-Asparagine motif found on the turn), and the Ω-loop (named after the shape of the loop). Abbreviations: Ala, alanine; Arg, arginine; Glu, glutamic acid; Gly, glycine; Ser, serine; Thr, threonine.

Extended-spectrum β-lactamases in group 1, which give low level resistance to cefotaxime or ceftazidime, possess amino acid substitutions that increase the ability of the enzyme to hydrolyse ceftazidime faster than cefotaxime. Groups 2 and 3 will produce high levels of resistance to both ceftazidime and/or cefotaxime. Enzymes in group 2 hydrolyse ceftazidime to a greater extent than cefotaxime, whereas group 3 enzymes hydrolyse cefotaxime better than ceftazidime. Group 4 contains those β-lactamases that hydrolyse the 3GCs and are resistant to inhibition by the β-lactamase inhibitors such as clavulanic acid. Often the *in vitro* ability to hydrolyse cefotaxime is greater than that to hydrolyse ceftazidime, but the fast penetration of cefotaxime ensures that this hydrolysis is insufficient to overcome the drug's antibacterial activity [20, 40, 72, 83].

The Payne & Amyes classification was the best system available to concisely describe the plasmid-mediated SHV- and TEM-*derived* β-lactamases at the start of this thesis. No account, however, is taken of the amino acid sequence of these closely related ESBL.

Group‡		Phenotype	Activity			Examples...
			CAZ	CTX	Clav	
1		Low 3GC resistance	++	+	-	TEM-E2, TEM-2, TEM-7, TEM-12
2		High 3GC resistance	++++	+++	-	TEM-E3, TEM-6, TEM-9, TEM-10
3	a	High 3GC resistance	++++	+++++	-	TEM-3, TEM-4, TEM-5, TEM-8
	b					SHV-2, SHV-3, SHV-4, SHV-5
	c					FEC-1, DJP-1
4		3GC resistance	++++	+++	++	MIR-1, BIL-1

Table 1.10: Summary of the Payne & Amyes classification of plasmid-mediated extended-spectrum β-lactamases

Abbreviations: 3GC, 3rd generation cephalosporins; CAZ, ceftazidime; Clav, clavulanic acid.
‡ Group 3 is subdivided into TEM-*derived* (a), SHV-*derived* (b), and those of unknown origin (c) according to the data available when the classification was devised Payne & Amyes [152].

10. CONCLUDING REMARKS

The evolution of the β-lactamases have been studied in detail by a number of workers since the enzymes discovery in 1940. The Ambler class A β-lactamases, specifically the ubiquitous SHV- and TEM-*type* enzymes, are of particular clinical importance and an understanding of the environmental pressures that have selected the enzymes is useful in the design of new β-lactam antimicrobial agents. The parental enzymes (TEM-1, TEM-2 and SHV-1) can be readily mutated to extended-spectrum enzymes in the laboratory environment when challenged with a 3GC [161, 162]. This phenomenon, however, proposes the question: " If the ESBL mutations occur so readily, why are the reported incidences of ESBL-mediated resistance to the 3GCs so few?"

The epidemic of ESBL-mediated resistance promised by certain groups [107, 153, 163-165] in the 1980s failed to materialise [152]. One of the proposed reasons for this was the limitation of 3GC use to hospitals. The introduction of orally active 3GCs has allowed an increase in the number bacteria to be challenged with this selective pressure. The oral 3GCs are no more likely to select ESBL-mutations than the parenteral 3GCs [166], but the incidence of 'challenge events' will increase as the drugs become more widely prescribed. A pool of 3GC resistant strains may then develop in the general population. The sporadic nature of reports describing ESBL, however, suggests that the TEM- and SHV-*derived* ESBL are somehow unstable compared to the parental enzymes. This phenomenon has not been investigated or explained.

Most researchers have either examined the clinical implications of ESBL or the effect of the ESBL-mutations on substrate binding. The clinical aspects of the ESBL have rarely been studied in combination with a detailed analysis of the molecular and biochemical characteristics of the ESBL-mutations. There has been no satisfactory explanation for the multiplicity of the TEM- or SHV-*derived* enzymes to date. Much of the research, like the combinations of the ESBL-mutations (Table 1.8 page 44, Table 1.9 page 45 and Figure 1.11 page 47), appears to have been random with respect to evolutionary considerations. Although this approach has been useful in elucidating the substrate binding of many class A β-lactamases, the contribution of each individual clinical mutation, separately or in clinically observed combinations, has

not been fully investigated. There are 23 SHV- and TEM-*derived* ESBL that have been shown to be genotypically and phenotypically distinct: the classification schemes described above (see Section 8 pages 29-41 & Section 9.2 pages 46-48) do little, unfortunately, to accommodate the inter-relationships of these closely related enzymes.

11. AIMS OF THIS THESIS

1. To evaluate the inter-relationships of the SHV- and TEM-*derived* β-lactamases, and to establish a rationale for the study of the ESBL.

2. To study the various TEM-*derived* ESBL enzymologically, to relate the enzymes structural variations to changes in function, and to elucidate the effects of the various point mutations present within the ESBL on the binding of the 7-oxyimino cephalosporins.

3. To identify the possible evolutionary course of events, promoted by the selective pressure applied through the clinical use of the 3GCs, that led to the selection of many of the ESBL.

4. To investigate the relative instability of the SHV- and TEM-*derived* ESBL genes compared to the parental TEM-1, TEM-2 and SHV-1 β-lactamase genes.

5. To relate the molecular and biochemical aspects of the SHV- and TEM-*derived* β-lactamases to the possible selective pressures applied by antimicrobial chemotherapy in the clinical setting.

Chapter 2

MATERIALS & METHODS

1. REAGENTS & ANTIMICROBIAL AGENTS

Chemicals and reagents were all supplied by Sigma Chemicals (Poole, Dorset) unless otherwise indicated. The antimicrobial agents were supplied by Sigma chemicals unless otherwise stated (Table 2.1 page 52). Solutions of antimicrobial agents prepared from non-sterile powders were filter sterilised through a 0.22μm pore membrane (Acrodisc 0.2μm sterile syringe filters; Gelman Sciences, Ann Arbor, Michigan, USA) prior to use. All solutions of antimicrobial agents were freshly prepared with sterile ultra-pure deionised water unless otherwise indicated.

2. BACTERIAL STRAINS

The standard bacterial strains used for the experiments that comprise this thesis are shown in Table 2.2 (page 53) and the standard vector plasmids are detailed in Table 2.3 (page 53). Details of strains expressing standard β-lactamases are listed in Table 2.4 (page 54).

Antimicrobial agent		Abbreviation used in text	Supplier
Generic name	Proprietary name		
Ampicillin	-	Amp	Sigma Chemicals
Amoxycillin	Amoxil	Amx	Bencard
Cefotaxime	Claforan	Ctx	Roussel Laboratories Ltd
Ceftazidime	Fortum	Caz	Glaxo Laboratories Ltd
Cefuroxime	-	Cfx	Sigma Chemicals
Cephaloridine	-	Cep	Sigma Chemicals
Chloramphenicol	-	Cm	Boehringer Mannheim UK
Clavulanic acid	-	Clav	Glaxo Group Research Ltd.
Kanamycin	-	Kan	Boehringer Mannheim UK
Nalidixic acid	-	Nal	Sigma Chemicals
Nitrocefin	-	Ncfn	Glaxo Group Research Ltd
Penicillin G	-	PenG	Sigma Chemicals
Rifampicin	Rifadin	Rif	Ciba Laboratories
Streptomycin	-	Sm	Sigma Chemicals
Tetracycline	Achromycin	Tet	Lederle Laboratories

Table 2.1: Antimicrobial agents

All strains were stored at -70°C in cryovials (Alpha Laboratories, Eastleigh, Hants.). The required strain was inoculated into nutrient broth and incubated, with shaking, overnight at 37°C. An aliquot of the overnight culture was thoroughly mixed with sterile glycerol to a final concentration of 10%V/v and immediately frozen at -70°C. Strains were sub-cultured directly from stock onto solid media, for verification of phenotype and purity prior to use, but were never passaged.

Strain	Genotype	Source	Reference
E. coli K12 J53-2	*pro*, *met*, Rif^R	S.G.B. Amyes	[167]
E. coli K12 J62-1	*pro*, *his*, *trp*, *lac*, Nal^R	S.G.B. Amyes	[167]
E. coli K12 J62-2	*pro*, *his*, *trp*, *lac*, Rif^R	S.G.B. Amyes	[167]
E. coli K12 TG1	*thi*, Δ(*lac-pro*AB), F'[$lacI^q$ *lac*ZΔM15]	Amersham International plc	[168]
E. coli DH5α	*thi*, Δ*lac*U169 (Φ80 *lac*ZΔM15)	Gibco BRL	[169]

Table 2.2: Standard bacterial strains

Plasmid	Size	Markers	Reference
pAT153	3.7-kb	Amp^R, Tet^R	[170]
pBGS18+	4.4-kb	Kan^R, *lac*Z'	[171]
pBR322	4.4-kb	Amp^R, Tet^R	[172]
pKK233-2	4.6-kb	Amp^R	[173]
pSG-2	4.4-kb	Kan^R	[174]
pSG-TEM-1	5.3-kb	Amp^R, Kan^R	[174]
pUC8	2.7-kb	Amp^R, *lac*Z'	[175]
pUC18	2.7-kb	Amp^R, *lac*Z'	[176]

Table 2.3: Details of vector plasmids

β-Lactamase	pI	Bacterial strain	Plasmid	Source§	Reference
OXA-2	7.7	*E. coli* J53-2	R46	SA319	[177]
SHV-3	7.0	*E. coli* J53-2	pUD16	DP191	[178-180]
TEM-1	5.4	*E. coli* JM108	pUC8	SA1489	[175]
		E. coli JM83	pUC18	SDB73	[176]
		E. coli TG1	pAT153	SDB101	[170]
		E. coli 1009	pBR322	SA473	[172]
		E. coli J53-2	R6K	SA471	[181]
		E. coli J62-2	R1	SA17	[182]
TEM-2	5.6	*E. coli* J62-2	RP4	DP43	[181]
TEM-3	6.3	*E. coli* J62-1	pCFF04	DP189	[180, 183]
TEM-5	5.5	*E. coli* J62-2	pCFF14	DP70	[180, 184]
TEM-6	5.9	*E. coli* J53-2	pMG226	DP314	[180, 185]
TEM-7	5.41	*E. coli* J62-1	pIF100	DP187	[180, 186]
TEM-9	5.5	*E. coli* J53-2	pMG228	DP41	[180, 187]
TEM-10	5.57	*E. coli* J53-2	pJPQ100	DP211	[188]
TEM-12	5.25	*E. coli* MG32	Chromosomal	SDB68	[189]
TEM-26	5.58	*E. coli* DH5α	pJPQ101	SDB157	[190]
TEM-E1	5.41	*E. coli* J53-2	pUK720	DP63	[141, 191]
TEM-E2	5.25	*E. coli* J53-2	pUK721	DP67	[141, 151]
TEM-E3	5.57	*E. coli* J53-2	pUK722	DP61	[141, 192, 193]
		E. coli J53-2	pUK723	DP315	[141, 192, 193]
TEM-E4	5.61	*E. coli* J53-2	pUK724	DP59	[141, 194]

Table 2.4: Bacterial strains producing standard β-lactamases

§ Source of host organism (culture collection number: DP prefix refers to D.J. Payne's culture collection; SA prefix refers to S.G.B. Amyes' culture collection; SDB prefix refers to S.K. Du Bois' culture collection).

3. GROWTH MEDIA

Unless otherwise stated all growth media, and components thereof, were sterilised by autoclaving for 15 minutes at 121°C. The standard working concentrations of antibiotic supplements for addition to both minimal and complex media are listed in Table 2.5.

3.1. MINIMAL MEDIA

Minimal salts medium was prepared as a double strength solution, as described by Davis & Mingioli [195] (double-strength DM) (Table 2.6 page 56). A single-strength minimal salts medium (single-strength DM) was prepared by dilution of double-strength DM with an equal volume of sterile distilled water. Single-strength DM was used for the dilution of bacterial cell suspensions. For the preparation of minimal salts growth media D-glucose was added to a final concentration of 0.28%^{W}/v, along with the necessary auxotrophic requirements (Table 2.7 page 56).

Antibiotic	Stock solution concentration‡	Solvent	Working concentration†
Ampicillin	50mg/ml	Water	50-100mg/l
Ceftazidime	10mg/ml	Water	2-8mg/l
Kanamycin	20mg/ml	Water	50mg/l
Nalidixic acid	2.0mg/ml	Water	20mg/l
Rifampicin	2.5mg/ml	Water	25-50mg/l
Tetracycline	5mg/ml	Ethanol	50mg/l

Table 2.5 : Antibiotics used for the selection of resistant *Escherichia coli* cells

‡ Solutions were freshly prepared and sterilised by filtration (Acrodisc 0.2µm syringe filter, Gelman Sciences). † Concentrations of antibiotics varied according to plasmid copy number and resistance mechanism.

Single-strength DM supplemented with glucose and thiamine is called MGT medium throughout this thesis, and was primarily used for the maintenance of *E. coli* TG1 strains.

Solid media was prepared by adding the required auxotrophic supplements, selective antibiotics, and glucose to a 50ml aliquot of double-strength DM and making up to 60ml. The enriched solution was then mixed with 40ml sterile molten Bacteriological Agar No. 1 (37.5g/l) (Oxoid, Basingstoke, Hants.) immediately prior to pouring the plates.

Ingredient	Formula	Double-strength	Single-strength
di-Potassium hydrogen phosphate	K_2HPO_4	14.0g/l	7.0g/l
Potassium dihydrogen phosphate	KH_2PO_4	6.0g/l	3.0g/l
tri-Sodium citrate	$Na_3C_6H_5O_7$	0.94g/l	0.47g/l
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	0.2g/l	0.1g/l
Ammonium sulphate	$(NH_4)_2SO_4$	2.0g/l	1.0g/l

Table 2.6: Davis & Mingioli minimal salts medium

Auxotrophic supplement	Stock solution concentration	Final concentration	Method of sterilisation
L-Histidine	5.0g/l	50mg/l	Steaming/30min.
L-Methionine	5.0g/l	50mg/l	Steaming/30min.
L-Proline	5.0g/l	50mg/l	Steaming/30min.
L-Tryptophan	2.0g/l	50mg/l	Steaming/30min.
Thiamine hydrochloride	1.0g/l	2mg/l	Filtration‡

Table 2.7: Supplements for minimal media

‡ Acrodisc 0.2µm syringe filter (Gelman Sciences)

3.2. COMPLEX MEDIA

The following complex media were used: Isosensitest Broth (CM473), Nutrient Broth No. 2 (CM67), Isosensitest Agar (CM471) and MacConkey Agar (CM7b), all supplied by Oxoid (Basingstoke, Hants.).The following media were also used (quantities for a final volume of one litre):

SOC MEDIUM: 20g Bacto tryptone (Difco, Detroit, Michigan, USA), 5g Bacto yeast extract (Difco), and 0.5g sodium chloride, with final concentrations of 25mM potassium chloride and 20mM glucose. The pH was adjusted to 7.0 with sodium hydroxide.

LURIA-BERTANI (LB) MEDIUM: 10g Bacto tryptone (Difco), 5g Bacto yeast extract (Difco), and 10g sodium chloride. LB Agar was prepared with the addition of 15g Bacteriological Agar No. 1 (Oxoid) to the medium before making the volume up to one litre. LB 'top agar' (for overlaying agar plates) was prepared as for LB broth except for the addition of 7g Bacteriological Agar No. 1 (Oxoid) per litre.

4. ANTIBACTERIAL SUSCEPTIBILITY TESTING

Minimum inhibitory concentrations (MICs) were performed on Isosensitest (IST) agar following the British Society for Antimicrobial Chemotherapy (BSAC) guidelines for susceptibility testing where applicable [196]. Antibiotic sensitivity discs were supplied by Mast Laboratories Ltd. (Merseyside). MICs were determined by agar double dilution of the antimicrobial agent. Agar, containing the appropriate concentrations of antimicrobial agent, was inoculated with a 2µl spot of each organism, delivered from a multipoint inoculator (Denley, Billinghurst, Surrey). Bacterial strains to be tested were inoculated into nutrient broth and incubated in a static environment for 12 hours at 37°C, before dilution in single-strength DM to give an inoculum of 10^4 colony forming units (cfu)/spot in each case (unless otherwise stated). Inoculated agar plates were incubated overnight at 37°C in stacks of no more than three Petri dishes. MICs of co-amoxiclav (amoxycillin/clavulanic acid ratio 2:1) are expressed in terms of the amoxycillin concentration.

5. TRANSCONJUGATIONAL TRANSFER OF PLASMIDS

The method of conjugal transfer of plasmids employed was as described by Amyes & Gould [197]. Broth cultures of donor and recipient strains were always incubated statically at 37°C. The donor and recipient strain were cultured overnight in nutrient broth, then 4.5ml of fresh, pre-warmed, nutrient broth was inoculated with 100µl of the donor strain and 1.0ml of the recipient strain, and gently mixed. The mixture was incubated at 37°C for six hours. The bacterial cells were then harvested using a Heraeus Labofuge 6000 by centrifugation at 4500rpm (3000×*g*) for 20 minutes. The cell pellet was resuspended in 5ml of single-strength DM and a series of ten-fold dilutions prepared. A 100µl aliquot of each dilution was spread onto minimal media containing the appropriate auxotrophic supplements and selective antibiotics. The inoculated plates were incubated at 37°C for 48 hours. All potential transconjugants were verified by checking the auxotrophic requirements and resistance markers.

To select ampicillin or ceftazidime resistant *E. coli* K12 J62-2 transconjugants, minimal media containing the auxotrophic requirements of this strain (proline, histidine, and tryptophan; see Table 2.7 page 56), plus 25mg/l rifampicin and 50mg/l ampicillin or 4mg/l ceftazidime, as appropriate for selection, was used.

6. PURIFICATION OF β-LACTAMASES

The three different methods of β-lactamase purification employed are described below. Each method was based on the initial disruption of the bacterial cells with ultrasound [97, 198]. A Sorvall RC-5B Refrigerated Superspeed Centrifuge (DuPont Instruments, Stevenage, Herts) was used for all the centrifugation described in this section, unless otherwise stated. The specific Sorvall fixed-angle rotor (SM-24, SA-600, or GS-3: all supplied by DuPont Instruments) used in the Sorvall RC-5B Centrifuge for each centrifugation step is stated where appropriate.

6.1. CRUDE, CELL FREE, β-LACTAMASE PREPARATIONS

Each bacterial strain investigated was streaked onto nutrient agar slopes and incubated overnight at 37°C. Cell growth was then washed off the surface of the nutrient agar slope with 1.0ml of 50mM sodium phosphate buffer (pH 7.0),

transferred to a fresh sterile container, and cooled on ice for 5 minutes. Bacteria were disrupted by sonication (MSE Soniprep 150, MSE Instruments, Crawley, Sussex) with the cell suspensions cooled on ice [198, 199]: two 30 second pulses of 7-8μm amplitude separated by a 30 second cooling period. The lysate was cleared of cell debris by centrifugation at 4°C in an MSE Micro Centaur centrifuge at 13000rpm (11600×*g*) for 20 minutes. The cell free supernatant was stored at -20°C until required.

ASSESSMENT OF β-LACTAMASE ACTIVITY ENZYME PREPARATIONS

The time, in seconds, for a 33μl aliquot of each β-lactamase preparation to change 100μl of a 100μM solution of nitrocefin from a yellow colour to red was taken as an indication of β-lactamase activity. Measurements were performed in a microtitre plate against a white background. This empirical assessment is referred to as the 'nitrocefin spot test' throughout this thesis.

6.2 PARTIAL PURIFICATION OF β-LACTAMASES

The required bacterial strain was inoculated into 5ml of LB broth and incubated at 37°C with vigorous shaking (~200rpm) for 6 hours. This seeder culture was added to 500ml of LB broth, in a 2l conical flask, which was then incubated overnight at 37°C with vigorous shaking (~180rpm). The overnight cell culture was harvested by centrifugation at 7500rpm (6000×*g*) for 15 minutes at 4°C in a GS-3 rotor. The resultant bacterial pellet was resuspended in 20ml of 50mM sodium phosphate buffer (pH 7.0), and re-pelleted at 4500rpm (3000×*g*) for 30 minutes at 4°C, with the SA-600 rotor. One ml of 50mM sodium phosphate buffer (pH 7.0) was added to the final cell pellet (~2ml in volume) and the cells resuspended to form a thick slurry. This sample was dispensed into ~1.5ml aliquots and suspended in an ice/water bath.

The cells were disrupted by sonication [198, 199] at 7-8μM amplitude (MSE Soniprep 150): three 30 second pulses separated by 30 second cooling periods. The separate aliquots were pooled and cooled on ice for 5 minutes. Nucleic acids were removed by precipitation with the addition of 0.1 volumes of 10%^{W}/v streptomycin sulphate. The cell lysate was cleared by centrifugation at 4°C

at 16000rpm (32000×*g*) for 1 hour with an SM-24 rotor. The supernatant was removed and used immediately, or stored at -20°C and thawed on ice before use.

PREPARATION OF A SEPHADEX G-75 GEL FILTRATION COLUMN

The method of column preparation was based upon that described by Andrews [200]. Fine grade Sephadex G-75 (Pharmacia, Uppsala, Sweden)[a] was swollen in an appropriate volume of 50mM sodium phosphate buffer, (pH 7.0) by maintainance at 100°C for 3 hours. The slurry was cooled to 4°C and poured into a $2cm^2 \times 90cm$ acrylic column (Amicon, Ltd, Stonehouse, Glos.) which was maintained at 4°C. When fully packed, an LKB 10200 Perpex peristaltic pump (Pharmacia) was connected, and the Sephadex was washed with 50mM sodium phosphate buffer (pH 7.0), at a flow rate of 0.2ml/minute for 48 hours. When not in use the Sephadex column was maintained by continuous recycling of a one to two litre reservoir of 50mM sodium phosphate buffer. After use the column was washed with a minimum of two column volumes (180ml) of 50mM sodium phosphate buffer (pH 7.0) before maintainance with continuous recycling of buffer.

CALIBRATION OF THE SEPHADEX G-75 GEL FILTRATION COLUMN

The Sephadex column was calibrated according to the method of Andrews [200]. Three proteins of known molecular weight (10mg/ml each of cytochrome C, chymotrypsinogen, and ovalbumin) were applied to the column in a 1ml aliquot and eluted at a rate of 0.2ml/minute in 50mM sodium phosphate buffer. Fractions were collected in 2ml aliquots with a LKB 2070 Ultrorac II fraction collector (Pharmacia). The elution volumes of the proteins were determined by measuring absorbance (λ = 280nm) of the fractions with a Perkin-Elmer UV/Vis Lambda 2 Spectrophotometer (Beaconsfield, Bucks.). Absorbance of each fraction was plotted against elution volume; this standard curve was used to estimate the elution volume of the β-lactamases passed through the column.

The total crude, cell free, lysates were applied to the Sephadex column and eluted with 50mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.2ml/minute, until

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[a] Dry bead diameter 10-40μm: Bed volume 12-15ml/g of dry Sephadex G-75: Recommended for the fractionation of peptides & globular proteins of a molecular mass between 3×10^3 and 7×10^4.

at least one column volume had been collected in 2ml fractions. Each fraction was subjected to a nitrocefin spot-test assay to ascertain the fractions containing the eluted β-lactamase. Fractions exhibiting peak β-lactamase activity were pooled and proteins precipitated by the addition of 70%W/v ammonium sulphate. The precipitated proteins were pelleted by centrifugation at 12000rpm (20000×*g*) at 4°C, in an SA-600 rotor for 30 minutes. The $(NH_4)_2SO_4$ precipitate was dispersed in 1.0ml of 50mM sodium phosphate buffer (pH 7.0) and the resultant slurry dialysed against 2l of 50mM sodium phosphate buffer (pH 7.0) at 4°C for 12 hours. The activity of each partially purified β-lactamase preparation was assessed with a nitrocefin spot test. Partially purified preparations were stored at -20°C until required.

6.3. PREPARATIVE ISOELECTRIC FOCUSING OF β-LACTAMASES

Preparative IEF of partially purified enzymes was required to separate individual β-lactamases of multiple β-lactamase producing strains, in order that the enzyme kinetics of the different β-lactamases could be determined. The method employed was modified from that described by Vecoli *et al.* [201] and Hood [202] for use with an LKB 2217 Ultraphor Electrofocusing unit (Pharmacia).

Agarose for Isoelectric focusing Type VIII (1.5g) was dissolved in 75ml sterile deionised water by heating to 121°C, followed by cooling to 55°C. Twenty ml of 50%W/v D-sorbitol, 2.5ml of 40%W/v carrier ampholytes pH 4-6, and 2.5ml of 40%W/v carrier ampholytes pH 3.5-10 were pre-warmed to 55°C and mixed with the molten agarose.

An LKB Gelbond film (for agarose gels) (Pharmacia) was placed onto the cooling plate of an LKB Ultraphor Electrofocusing unit. The cooling plate was warmed to 45°C by connecting the unit to a thermostatic circulator and the molten IEF agarose solution poured evenly onto the Gelbond film. The unit was then cooled to 15°C, and agarose solidified, prior to the application of electrode strips, soaked in the appropriate buffer (1M H_3PO_4 for the anode and 1M NaOH for the cathode), close to the short edges of the gel. One ml of a partially purified β-lactamase preparation was applied evenly to the surface of the agarose, to within 10mm of the gel edges and electrode strips. The β-lactamases were focused for 3 hours at

15°C at a constant power of 20 Watts (Power supply settings: voltage = 1500V, current = 30mA, power = 20W). The two edges of the agarose gel between the electrode strips, were visualised by overlaying the gel with strips of filter paper soaked in 1mM nitrocefin solution. When the regions of β-lactamase activity were identified the focused enzyme bands were excised. One ml of 50mM sodium phosphate buffer (pH 7.0) was added to each agarose gel slice (between 5-10mm in width), after slicing into smaller fragments, and dialysed overnight against 2l of 50mM sodium phosphate (pH 7.0) at 4°C. The agarose fragments were then pelleted at 16000rpm (32000×*g*) at 4°C in an SM-24 rotor. The supernatant was removed and was stored at -20°C for up to one month prior to use. The activity of each preparation was estimated with a nitrocefin spot test. Purity of preparations were verified by analytical IEF (see Section 7 below).

7. ANALYTICAL ISOELECTRIC FOCUSING

β-Lactamases were identified by analytical IEF as described by Matthews *et al.* [105]. The β-lactamase preparations were focused on horizontal thin layer polyacrylamide gels [203] containing broad range carrier ampholytes of pH 3.5-10, or a mixture of broad range (pH 3.5-10) and narrow range (pH 4-6) carrier ampholytes (ratio 1:1).

Thin layers of polyacrylamide gel, 200m × 150mm × 1mm, were prepared by polymerisation of the gel solution, described in Table 2.8 (page 63), catalysed by riboflavin [204] in the presence of ultra-violet light between two glass plates 1mm apart. To promote adhesion of the polyacrylamide gel to one of the two glass plates, it was necessary to coat one glass plate with a binding solution. The remaining glass plate was siliconised, to reduce adhesion, with Sigmacote silconising solution.

The binding solution was prepared by dissolving 0.5%W/v gelatine (ca. 225 bloom from calf skin: Aldrich Chemical Co. Ltd., Gillingham, Dorset) and 0.5%W/v chromium potassium sulphate dodecahydrate (Aldrich Chemical Co. Ltd.) in sterile distilled water. Glass plates (210mm × 160mm) were submersed in the coating solution for 10 minutes, before removal from the solution and air-drying for 1 hour prior to use.

Samples of β-lactamase preparations were loaded close to the anode on the surface of the gel (volumes in μl equivalent to the nitrocefin spot test time, up to a maximum of 50μl were applied). In all cases β-lactamases of known isolectric point (see Table 2.4 page 54) were focused alongside novel enzymes.

Analytical IEF gels were focused at a constant power of 1 Watt overnight at room temperature (Power supply settings: voltage = 500V, current = 20mA, power = 1.0W). The focused β-lactamase bands were visualised by overlaying the polyacrylamide gel with sheets of filter paper soaked in 1mM nitrocefin [104]. Isoelectic points of unknown or novel enzymes were estimated from the focused bands of enzymes of known pI.

8. ENZYME KINETICS

The SHV- and TEM-*type* β-lactamases catalyse β-lactam hydrolysis (Figure 2.1 page 64) by a double displacement mechanism, via an acyl-enzyme intermediate [205, 206] that obeys the principles of Michaelis-Menten kinetics [137, 207] (Figure 2.2 page 64). The progress of enzyme catalysed reactions could be monitored by measuring the change in absorption of light (A) over time [104, 208, 209].

Ingredient (stock solution)	Volume (ml)	Final concentration	Supplier
Sterile distilled water	25.0	-	-
5%$^{V}/_{v}$ *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED)	0.2	0.05%$^{V}/_{v}$	Sigma Chemicals
40%$^{W}/_{v}$ carrier ampholytes: pH 3.5-10 pH 3.5-10/pH 4-6 (Ratio 1:1)	2.0	2.0%$^{W}/_{v}$	Sigma Chemicals
33.9%T, 2.7%C acrylamide/ *N,N'*-methylene *bis*-acrylamide	9.0	7.6%T, 2.7%C	BDH Chemicals
0.002%$^{W}/_{v}$ riboflavin	4.0	0.0002%$^{W}/_{v}$	Sigma Chemicals

Table 2.8: Composition of analytical IEF gels

All spectrophotometric studies of β-lactamases were performed on either a Perkin-Elmer UV/Vis Lambda 2 Spectrophotometer or a Perkin-Elmer UV/Vis 554 Spectrophotometer. All assays were carried out at 37°C in 50mM sodium phosphate buffer (pH 7.0). Substrate solutions were prepared in 50mM sodium phosphate buffer (pH 7.0).

The wavelength (λ) at which enzymatic-degradation of a given β-lactam substrate was measured, was that which gave the maximum difference in absorbance between unhydrolysed and hydrolysed substrate. Details of the wavelength used for β-lactamase assays of the various β-lactam substrates (λ_{max}) is given in Table 2.9 (page 65).

8.1. MEASUREMENT OF β-LACTAMASE ACTIVITY

The rate of hydrolysis for a fixed concentration of substrate (1mM for penams, 0.1mM for cephems) was measured to give an indication of enzyme activity. An

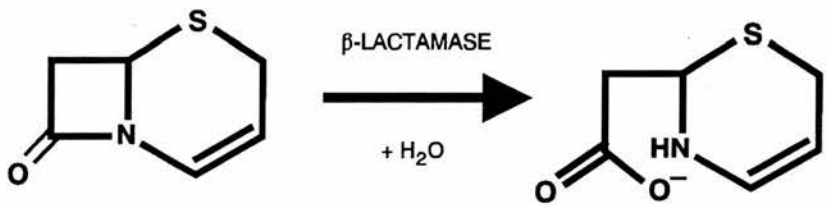

Figure 2.1: Hydrolysis of the β-lactam bond of cephems

Enzymatic degradation of the β-lactam antimicrobial agents by β-lactamases adds a water molecule across the β-lactam bond.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\bullet}S \xrightarrow{k_2} E{-}S \xrightarrow{k_3} E + P$$

Figure 2.2: The mechanism of β-lactamase hydrolysis of β-lactam antimicrobial agents

The enzymatic hydrolysis of β-lactam antimicrobial agents follows Michaelis-Menten kinetics. Abbreviations: E, enzyme; S, substrate; E•S, noncovalent Michaelis complex; E-S, acyl-enzyme complex; P, ring opened product; k_n, rate constants. From Bush & Sykes (1986) [137].

aliquot of a β-lactamase preparation was added to a test cuvette containing the substrate, and the decrease in absorbance over time (in minutes), was measured with reference to a blank solution containing the same concentration of enzyme but no substrate. Rate of hydrolysis was defined as micromoles of substrate hydrolysed per minute, per millilitre of enzyme preparation [104]:

$$R = \frac{\Delta A \times n \times d}{A_0 \times t}$$

where: R = initial rate of hydrolysis (μmoles/minute/ml of enzyme)
ΔA = change in absorbance over time, t (minutes)
n = number of micromoles of substrate added to cuvette
d = factor adjusting enzyme volume to 1ml
A_0 = absorbance of intact substrate solution at $t = 0$

The change of absorbance over time was measured for the initial 10% of the degradation reaction, where ΔA remained proportional to time. The values obtained by this method were normalised with respect to ampicillin or nitrocefin, to obtain the 'relative rate of hydrolysis' for each substrate, as proposed by Bush & Sykes [137].

β-Lactam compound	Molecular Mass‡	Wavelength (λ_{max})
Ampicillin	371.4	238nm
Cefotaxime	477.4	265nm
Ceftazidime	546.6	260nm
Cefuroxime	446.4	260nm
Cephaloridine	415.5	255nm
Nitrocefin	516	384nm
Penicillin G (benzylpenicillin)	356.4	238nm

Table 2.9: Optimum wavelengths for the measurement of β-lactam hydrolysis

‡ Molecular mass of sodium salt, except for nitrocefin (free base).

8.2. DETERMINATION OF ENZYME KINETIC PARAMETERS

The initial rate of hydrolysis (v_0) for a range of substrate concentrations (S), at which the Beer-Lambert law was obeyed, were determined photometrically. The results were analysed by the method of Lineweaver & Burke [210] and the maximum rate of reaction (V_{max}) and the Michaelis constant (K_m) were calculated from a plot of $1/v_0$ against 1/S. The efficiency of hydrolysis was calculated by dividing the V_{max} by the K_m [75, 211].

The I_{50} value was defined as the concentration of inhibitor required to inhibit enzyme activity by 50%. The initial rate of hydrolysis of 1mM penicillin G was determined in the absence of inhibitor. The β-lactamase inhibitor clavulanic acid was incubated with enzyme for 5 minutes at 37°C, before initiation of the reaction by addition of penicillin G to a final concentration of 1mM. The percentage inhibition for clavulanic acid concentrations increasing from 10nM to 1mM were determined. Percentage inhibition was then plotted against the logarithm (to the base 10) of the inhibitor concentration, and the I_{50} value determined by interpolation.

9. THE ISOLATION AND PURIFICATION OF DNA

A number of different methods were used for the isolation of DNA depending on its nature, or source, and the proposed use of the isolated DNA.

9.1. ISOLATION OF SMALL BACTERIAL PLASMIDS

Small molecular weight plasmids (2-kb to 10-kb) were isolated from cultures of *E. coli* by a procedure based on the 'Rapid boiling method' of Holmes & Quigley [212]. Bacteria from a small volume (1-1.5ml) of an overnight LB broth culture, were harvested and suspended in STET buffer (8%W/v sucrose, 50mM Tris·Cl pH 8.0, 50mM EDTA, 5%V/v Triton X-100), and boiled for 1 minute in the presence of 1%W/v lysozyme. An insoluble clot of cell debris and genomic DNA was removed by centrifugation (13000rpm (11600×*g*) for 10 minutes in an MSE Micro Centaur centrifuge). Plasmid DNA was recovered from the supernatant by precipitation with an equal volume of isopropranol at -70°C for 10 minutes. The precipitate was collected by centrifugation (9500×*g* for

10 minutes) and resuspended in a 300mM sodium acetate solution (pH 5.5), followed by a repeat centrifugation step to remove any remaining insoluble material. Plasmid DNA was precipitated at -70°C for 30 minutes after the addition of two to four volumes of ethanol. The precipitated plasmid DNA was pelleted by centrifugation (11600×*g* for 10 minutes), washed in a small volume of ethanol, and dried under vacuum for 10-20 minutes. The dried DNA-pellet was resuspended in 20µl of TE buffer (10mM Tris·Cl pH 7.5, 0.1mM EDTA).

This method was used for the isolation of cloning vector plasmids and double-stranded M13 phage DNA.

9.2. ISOLATION OF LARGE BACTERIAL PLASMIDS

Overnight LB broth cultures (4.5ml) were harvested by centrifugation for 30 minutes at 3100rpm (2000×*g*) in a Sorvall RT6000D Refrigerated Centrifuge with H1000B rotor (DuPont Instruments). A modification of the alkaline lysis method described by Takahashi & Nagano [213] was used to isolate large bacterial plasmids of clinical origin. The harvested cells were suspended in TAER buffer (50mM Tris-acetate pH 8.0, 10mM EDTA, 100µg/ml RNase A) and lysed at room temperature with the addition of an equal volume of 200mM NaOH/1%^{W}/v sodium dodecyl sulphate (SDS) for 5 minutes. An additional aliquot (equal to half the total volume) of 3M sodium acetate (pH 5.5) was added to neutralise the lysing solution. The insoluble clot of cell debris and genomic DNA was removed by centrifugation (13000rpm (11600×*g*) in an MSE Micro Centaur centrifuge at 4°C). The supernatant was extracted with an equal volume of chloroform, before precipitation of the plasmid DNA on ice for 10 minutes after the addition of two volumes of ehtanol. The precipitate was pelleted by centrifugation (11600×*g* for 10 minutes at 4°C), washed in a small volume of ethanol, and dried under vacuum for 10 minutes. The dried DNA pellet was redissolved in 20-50µl of an appropriate buffer.

DNA extracted by this modified alkaline lysis method was typically used to isolate plasmids up to ~150-kb in size, and often subjected to restriction analysis (see Section 11.2 page 73).

9.3. Isolation of total genomic DNA

Genomic DNA was isolated by an SDS-proteinase K treatment of *E. coli* cells similar to that described by Towner [214]. DNA prepared by this method was added to polymerase chain reactions as template DNA.

Cells were harvested from 1.5ml of an overnight LB broth culture, centrifuged (11600×*g* for 10 minutes in an MSE Micro Centaur centrifuge), and resuspended in 200µl of TESUC buffer (50mM Tris·Cl pH 8.0, 10mM EDTA, 7%^{W}/v sucrose). Forty µl of 0.1%^{W}/v lysozyme and 20µl of 0.2%^{W}/v proteinase K were added and the sample incubated at 37°C for 30 minutes. SDS, to a final concentration of 1%^{W}/v, was added, followed by a second incubation for 30 minutes at 37°C. The sample was then extracted five times with an equal volume of phenol/chloroform/isoamyl alcohol (ratio 25: 24: 1, saturated with TESUC buffer). Two volumes of ice-cold ethanol were added to the sample and the precipitate collected by centrifugation (11600×*g* for 10 minutes). The DNA was washed in ethanol and the pellet air-dried for 20-30 minutes. The DNA was redissolved in 50µl of TE buffer (pH 8.0).

9.4. Isolation of single-stranded M13 phage template DNA

Single-stranded template M13-DNA was prepared for use in site-directed mutagenesis experiments, or DNA sequencing reactions, by a modification of the polyethylene glycol (PEG) precipitation methods, described in the 'M13 cloning and sequencing handbook' (Amersham International plc [168]) and by Howe & Ward [215].

A single colony of *E. coli* TG1 (plasmid and phage free) was inoculated into 50ml MGT media (single-strength DM medium supplemented with thiamine and glucose at the concentrations described in Section 3.1 page 55) and incubated overnight at 37°C with vigorous shaking (~180rpm). An aliquot (100µl) of the overnight culture was inoculated into 50ml of LB broth and incubated at 37°C with vigorous shaking (~180rpm) for 1-2 hours until cell growth was just visible ($OD_{550} \approx 0.3$). Two hundred µl of phage-containing supernatant, or purified M13 phage DNA, was added to the *E. coli* TG1 culture and incubated at 37°C for 5-8 hours with vigorous shaking (~180rpm). The culture was then cooled on ice for 5 minutes,

prior to pelleting the bacterial cells by centrifugation at 12000rpm (20000×*g*) at 4°C, in a Sorvall RC-5B centrifuge with an SA-600 rotor, for 15 minutes. The single-stranded M13 phage DNA, present in the cell free supernatant, was precipitated for 30 minutes on ice by the addition of PEG 6000 and sodium chloride to final concentrations of 5%^{W}/v and 500mM respectively [216]. The precipitate was collected by centrifugation for 15 minutes at 8000rpm (8000×*g*) in a Sorvall RC-5B centrifuge with an SM-24 rotor. The DNA pellet was resuspended in TES buffer (20mM Tris·Cl pH 8.0, 10mM NaCl, 0.1mM EDTA) and extracted once with an equal volume of phenol (saturated with TE buffer), once with an equal volume of phenol/chloroform (ratio 1:1, saturated with TE buffer), and three times with an equal volume of isoamyl alcohol/chloroform (ratio 1:24, saturated with TE buffer). The single-stranded DNA was then precipitated at -70°C for 90 minutes by the addition of one-tenth of a volume of 3M sodium acetate (pH 5.5) and two volumes of ethanol. The precipitate was pelleted by centrifugation at 13000rpm (9500×*g*) at 4°C for 8 minutes (MSE Micro Centaur centrifuge). The pellet was washed in ethanol and dried under vacuum for 10-20 minutes. The dried DNA pellet was redissolved in 50µl of TE buffer (pH 8.0).

9.5. PREPARATION OF DOUBLE-STRANDED PLASMID DNA FOR CLONING AND DNA SEQUENCING REACTIONS

Double-stranded plasmid DNA was prepared with the Qiagen Plasmid Midi Preparation Kit (Qiagen, Chatsworth, California, USA). DNA yields of between 50 and 100µg were obtained from overnight LB broth cultures of 100-150ml. Bacteria were lysed by an alkaline-lysis method (similar to that described by Takahashi & Nagano [213]) with the reagents supplied with the Qiagen kit. Plasmid DNA was isolated by the selective nucleic acid binding characteristics of the Qiagen-tip 100 (a disposable modified anion-exchange resin column). Double-stranded DNA obtained by this method was reputed by the manufacturer to be equivalent in purity to that obtained by caesium chloride density gradient purification (as described by Sambrooke, Fitsch & Maniatis [217]).

The number of vector plasmids per chromosome were increased, when required for plasmid preparation, by the addition of chloramphenicol (34mg/ml in ethanol) to a

final concentration of 170mg/l after ~8 hours of incubation of the cultures at 37°C (with vigorous shaking). Cultures were then incubated for a further 8-12 hours at 37°C with vigorous shaking (~200rpm) before harvesting.

9.6. ISOLATION OF DNA FROM AGAROSE GEL SLICES

The Geneclean II Kit (Bio 101 Inc., La Jolla, California, USA) was used to recover DNA fragments from agarose gels (see this chapter Section 11.1 page 72). The protocol supplied by the manufacturer, based on the findings of Vogelstein & Gillespie [218], was followed throughout. Bands of DNA fragments were excised from agarose gels (0.7-1.5%W/v) after electrophoresis for separation purposes. DNA fragment purification by this method relied on the selective adsorbtion of DNA to the surface of small beads of a silica matrix (Glassmilk) supplied with the kit. DNA was eluted from the Glassmilk beads into 10µl of TE buffer.

9.7. THE ASSESSMENT OF CONCENTRATION & PURITY OF DNA

The absorbance of DNA solutions was measured at 260nm (A_{260}) and 280nm (A_{280}) with a Perkin-Elmer UV/Vis Lambda 2 Spectrophotometer, according to the method described by Towner [214]. One absorbance unit at 260nm was taken to be equivalent to a concentration of 50µg/ml of double-stranded DNA and 35µg/ml of single-stranded DNA. The ratio of the A_{260} and A_{280} value provided an estimation of purity: a ratio A_{260}/A_{280} of 1:1.8 indicated pure double-stranded DNA free from contaminating protein.

10. AMPLIFICATION OF DNA WITH THE POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) amplification of the open-reading frame (ORF) was based on the method provided by Disney & Dove [174]. Each PCR reaction was performed in a total volume of 100µl (Table 2.10 page 71) prepared in a 0.5ml polypropylene microcentrifuge tube (Alpha Laboratories) with a Techne PHC-2 Dri-Block Cycler (Cambridge, Cambs.). Reaction mixtures were overlaid with one or two drops of mineral oil to reduce evaporation, and the tips of the tubes were smeared with a thin film of silicone grease to improve thermal contact with the heating block.

Each reaction was then subjected to between 21 and 25 cycles, each of three steps (denaturation, annealing, and extension) as defined in Table 2.11 (page 72).

After completion of the PCR reaction (~2.5 hours) samples were placed on ice to 'quench' any further reaction. PCR reactions were then ethanol precipitated before verification of DNA amplification by agarose gel electrophoresis. PCR-amplification of TEM ORFs generated DNA fragments of ~900-bp in size. The biotinylated primer, Biotin-Bla 4', was used for the amplification of DNA for use with the Dynabeads M-280 Streptavidin (Dynal, Norway) direct sequencing protocol (see Chapter 3 below) or for use as DNA probes in Southern hybridisation procedures (see Section 11.4 page 75).

Component	Quantity	Supplier
10×*Taq* reaction buffer (Mg^{2+} free)	10µl	Promega (Southampton, Hants.)
25mM magnesium chloride	10µl	Promega
4mM dNTP stock solution‡	5µl	Boehringer Mannheim UK
Bla 3' PCR primer§	10pmoles	Glaxo Group Research Ltd.
Bla 4' PCR primer or Biotin-Bla 4' PCR primer†	10pmoles	Glaxo Group Research Ltd.
Genomic DNA preparation or a Washed *E. coli* cell suspension	1-2µl	-
Taq DNA polymerase	1 unit	Promega
Deionised distilled water	to 100µl	-

Table 2.10: PCR reaction components

‡ 4mM dNTP stock solution: 1mM dATP, 1mM dTTP, 1mM dGTP, 1mM dCTP in 10mM Tris·Cl (pH 7.5). § Bla 3' PCR primer: 5'-CTC TCT AGA AAA AGG AAG AGT ATG ATT-3'. † Bla 4' & Biotin-Bla 4' PCR primers: 5'-CTC GCA TGC GTA AAC TTG GTC TGC CAG TTA-3'.

11. THE ANALYSIS OF DNA

11.1. GEL ELECTROPHORESIS OF DNA

Intact plasmid DNA, restriction endonuclease digested plasmid DNA, or PCR-amplified DNA species were separated at neutral pH values in 0.7-1.5%W/v agarose gels in TAE buffer (40mM Tris-acetate pH 7.6, 1mM EDTA). Under these conditions duplex-DNA is negatively charged, hence loaded near the cathode, and migrates towards the anode when an electric field is applied [219]. Horizontal slab gel electrophoresis was carried out in a Bethesda Research Laboratories Horizon 20·25 gel tank (Life Technologies Inc, Petersburg, Florida, USA) or a Pharmacia GNA-100 Mini tank at constant voltage (between 50V for 12 hours and 200V for 1 hour, depending on the experiment and apparatus). λ phage-DNA digested with *Hind* III (Sigma Chemical Co.) was run alongside samples during electrophoresis, as an indicator of molecular weight of linear duplex-DNA. Each sample was mixed with one-sixth of a volume of GBX loading buffer (30%V/v glycerol, 0.25%W/v bromophenol blue, 0.25%W/v xylene cyanol) [217] prior to loading on to the gel. The DNA was visualised after electrophoresis by staining for one hour in a 50μg/l ethidium bromide solution and viewed on a UV transilluminator (UV Products, Cambridge).

Segment	Temperature	Time	Ramp rate	Repeats	Function
1	96°C	30 sec	48°C/min	×1	DNA denaturation
	50°C	60 sec	10°C/min		Primer annealing
	72°C	90 sec	30°C/min		Primer extension
2	96°C	15 sec	48°C/min	×20-24	DNA denaturation
	50°C	30 sec	10°C/min		Primer annealing
	72°C	90 sec	30°C/min		Primer extension
3	72°C	5 min	-	×1	Final extension

Table 2.11: The PCR heating cycle protocol

11.2. RESTRICTION ENDONUCLEASE DIGESTS

The restriction endonucleases utilised for the digestion of DNA in this thesis are given in Table 2.12. Plasmid DNA was digested with 5-10 units of enzyme in volumes of 10-30µl, with the manufacturer's recommended incubation buffer, at 37°C for between one and three hours depending on the purity of DNA and the efficiency of the specific enzyme.

11.3. DOT-BLOT HYBRIDISATION OF M13 VECTOR DNA

The dot-blot hybridisation procedure followed was obtained from Mrs Erica Christadoulou (Glaxo Group Research Ltd, Greenford, Middx). The method required the use of a NEN GeneScreen hybridisation transfer membrane (DuPont Instruments) in conjunction with the Schleicher & Schuell Minifold II dot-blot manifold (Anderman & Co. Ltd., Kingston-upon-Thames, Surrey). The apparatus was assembled according to the manufacturer's instructions.

Bacteria infected with the M13-phage were incubated overnight in 2ml of LB broth, before the phage containing supernatant was cleared by centrifugation at

Restriction endonuclease	Recognition sequence	Supplier
Alf II	CTTAAG	Boehringer Mannheim UK
*Bam*H I	GGATCC	Gibco BRL
Bgl II	AGATCT	Gibco BRL
*Eco*R I	GAATTC	Gibco BRL
Hind III	AAGCTT	Gibco BRL
Pst I	CTGCAG	Pharmacia
Sca I	AGTACT	Northumbria Biologicals Ltd
Sph I	GCATGC	Boehringer Mannheim UK
Xba I	TCTAGA	Boehringer Mannheim UK

Table 2.12: Restriction endonucleases

13000rpm (11600×*g*) for 10 minutes (MSE Micro Centaur centrifuge). Fifty µl of each phage containing supernatant was pipetted into wells of the Minifold II and allowed to pass through the hybridisation membrane. Up to 66 samples were applied to the membrane, together with three positive and three negative control samples. After all the samples had been applied and passed through the membrane the hybridisation filter was removed from the apparatus. The GeneScreen membrane was soaked in a denaturing solution (0.5M NaOH, 2.5M NaCl) for 15 minutes, before neutralisation in 3M sodium acetate (pH 5.5) for 15 minutes. The membrane was carefully blotted dry and the DNA cross-linked to the membrane by UV irradiation (Auto-crosslink setting, total energy = 1200 joules; Stratalinker, Stratagene, La Jolla, California, USA).

The membrane was incubated with an aqueous pre-hybridisation solution (0.2%^{W}/v bovine serum albumin (BSA), 0.2%^{W}/v Ficoll 400, 0.2^{W}/v polyvinylpyrrolidine (PVP), 1%^{W}/v SDS in 2×SSC buffer[a]) at 37°C for one hour. DNA probes were prepared by labelling the oligonucleotide primers (manufactured by Glaxo Group Research Ltd.) used for the SDM experiments (listed in Chapter 3): a solution containing 10pmoles of oligonucleotide DNA, 50mM Tris·Cl (pH 7.6), 10mM magnesium chloride, 5mM dithiothreitol (DTT), 0.1mM spermidine, 0.1mM EDTA (pH 8.0), 1 unit of polynucleotide kinase, and 1µl of ^{32}P-γ-ATP (370MBq/ml, 3000Ci/mmole: Amersham International plc) was prepared and incubated at 37°C for 30 minutes. The membrane was then hybridised with the DNA probe in fresh pre-hybridisation solution for 60 minutes at 37°C.

The membrane was initially washed with 2×SSC/0.1%^{W}/v SDS at 37°C for 5 minutes. The filter was washed with increasing stringency (decreasing salt concentration and increasing temperature) and monitored with a Geiger-Müller tube, until negative control samples gave significantly fewer counts than the positive controls. The membrane was then autoradiographed, by exposure to Hyperfilm MP X-ray film (Amersham plc), overnight at -70°C. The X-ray film was then developed with an automatic film processor.

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[a] 20×SSC buffer: 3.0M sodium chloride, 0.3M *tri*-sodium citrate.

11.4. SOUTHERN BLOTTING

Plasmid DNA was digested with restriction endonucleases and the resulting DNA fragments were separated by agarose gel electrophoresis in 1×TAE buffer at 50V for 12 hours. DNA was then transferred onto Hybond-C Extra membrane (Amersham International plc) by a capillary blotting method, based upon the procedure described by Southern [220], recommended by the manufacturer. After overnight blotting the membrane was baked at 80°C under vacuum for two hours.

DNA probes were prepared by the PCR-amplification (see Section 10 page 70) of the TEM-1 β-lactamase gene (*bla*T-1) ORF, with one of the two PCR-primers biotinylated (Biotin-Bla 4', see Section 10 page 70).

Prior to hybridisation of the DNA fragments with a biotin-labelled *bla*T-1 probe, the membrane was incubated in Gibco BRL Hybridisation bags with a

Ingredient	Pre-hybridisation solution	Hybridisation solution
Deionised formamide§	50%V/v	%V/v
SSC buffer	5×	5×
Denhardt's solution‡	5×	1×
Sodium phosphate buffer (pH 6.5)	50mM	20mM
SDS	0.1%W/v	0.1%W/v
Sheared herring sperm DNA†	0.1%W/v	0.05%W/v
Sodium dextran sulphate	-	5%V/v

Table 2.13: Pre-hybridisation and hybridisation solutions for Southern blotting

All solutions were prepared with deionised distilled water. § Deionised before use with Amberlite Monobed resin M.B.1 (BDH Merck, Poole, Dorset). ‡ 100×Denhardt's solution: 2%V/v BSA, 2%V/v Ficoll 400, 2%V/v PVP in 3×SSC buffer. † Boiled for 10 minutes, then cooled on ice for 10 minutes immediately prior to addition to solutions.

pre-hybridisation solution (Table 2.13 page 75) for 2 hours at 42°C. The pre-hybridisation solution was replaced with the hybridisation solution (Table 2.13) containing probe DNA (denatured by boiling for 10 minutes, and cooled on ice for 10 minutes immediately prior to use). Hybridisation was carried out at 42°C overnight.

The membrane was washed twice with 2×SSC/0.1%^{W}/v SDS for three minutes at room temperature, twice with 0.2×SSC/0.1%^{W}/v SDS for three minutes at room temperature, and twice with 0.16×SSC/0.1%^{W}/v SDS for 15 minutes at 50°C. The Hybond-C Extra membrane was finally washed briefly with 2×SSC at room temperature. Positive hybridisation signals were then visualised by means of the BluGene biotin detection kit (Gibco BRL), following the manufacturer's recommended procedure.

11.5. DNA SEQUENCING

DNA was sequenced with the USB Sequenase Version 2.0 DNA Sequencing Kit (Cambridge BioScience, Cambs.), based on the chain-termination method of Sanger *et al.* [221]. The Sequenase Version 2.0 polymerase is a genetic variant of T7 DNA polymerase lacking all 3'→5' exonuclease activity [222, 223]. Sequencing reaction products were radiolabelled with ^{35}S-α-dATP or ^{33}P-α-dATP (Amersham International plc), and separated by electrophoresis in a denaturing polyacrylamide gel (8%T, 5%C acrylamide/*bis*-acrylamide, 7M urea) with the Sequi-Gen Nucleic Acid Sequencing Cell (BioRad, Watford, Herts.), in an electrolyte gradient [224]. The recommended USB Sequenase Version 2.0 Kit protocols [225] for the sequencing of single-stranded template DNA (see Section 9.4 page 68 for preparation) and double-stranded plasmid DNA (see Section 9.5 page 69 for preparation) were followed. Other sequencing methods were employed [226-228] for specific purposes, and are described in more detail under the relevant section in Chapter 3. The details below refer to the preparation and running of a 210mm × 500mm × 0.4mm polyacrylamide gel.

The Sequi-Gen Cell was cleaned and assembled according to the manufacturer's recommendations: the integral/plate chamber (IPC) and outer glass plate were sequentially cleaned with deionised water, ethanol, chloroform, ethanol and finally

deionised water, to remove all traces of acrylamide and grease. The IPC was siliconised with Sigmacote prior to each experiment. The two 0.4mm spacers required for assembly were thoroughly cleaned with deionised water.

The 8%T, 5%C acrylamide/7M urea solution required for the preparation of the denaturing polyacrylamide gel is described in Table 2.14. The bottom of the electrophoresis gel was sealed by impregnating a Sealing Strip (BioRad) with the plug solution (Table 2.15). The polyacrylamide gel (Table 2.15) was poured, with the apparatus at ~20° to the horizontal, and left to polymerise for one hour.

Ingredient	Stock solution conc.	Supplier
Urea	7M	Sigma Chemicals
10×TBE buffer‡	1×	Sigma Chemicals
40%T, 5%C acrylamide/ *N,N'*-methylene-*bis*-acrylamide	8%T, 5%C	Sigma Chemicals

Table 2.14: Denaturing acrylamide/*bis*-acrylamide stock solution

All solutions were prepared with deionised distilled water. ‡ 10×TBE buffer: 0.89M Tris-borate (pH 8.3), 20mM EDTA.

Component	Plug solution§	Gel solution†
8%T, 5%C acrylamide/ *bis*-acrylamide, 7M urea stock solution	20ml	55ml
25%$^{W}/_{v}$ ammonium persulphate	140µl	220µl
TEMED	50µl	7µl

Table 2.15: Recipes for the denaturing polyacrylamide gel

§ Polymerisation time ~10 minutes. † Polymerisation time 30-60 minutes.

The Sequi-Gen unit was mounted in the Universal Base (lower buffer chamber), the IPC filled with 0.5×TBE, and the lower buffer chamber filled with 400ml of 1×TBE buffer. The polyacrylamide gel was pre-run for 30-40 minutes at constant power (Power supply settings: voltage = 3000V, current = 50mA, power = 60W) to warm the apparatus to the required running temperature of 50-55°C. Samples were loaded and the gel run for 1 hour before the addition of 200ml of 3M sodium acetate (pH 5.5) to the lower buffer chamber. Electrophoresis was continued at 60 Watts for a further two to three hours.

The polyacrylamide gel was removed from the apparatus and soaked in a solution of 10%V/v methanol/10%V/v acetic acid for 45 minutes before drying at 80°C for two hours under vacuum in a flat-bed gel drier. Autoradiography was carried out by exposure to Hyperfilm MP X-ray film (Amersham International plc) at room temperature for 12-48 hours, before developing the film in an automatic film processor.

12. CLONING OF DNA FRAGMENTS

12.1. LIGATION OF DNA

The vector and target DNA were digested with the appropriate restriction endonucleases (see Section 11.2 page 73) and the fragments separated by agarose gel electrophoresis (see Section 11.1 page 72). The required DNA fragments were excised from the agarose gel and purified with the Geneclean II Kit (see Section 9.6 page 70). The target DNA fragment and vector DNA fragment (in excess) were diluted in 20µl of Ligation buffer (Gibco BRL) with 0.1 units of T4 DNA ligase (Gibco BRL). Ligation was carried out for 12 hours at 16°C, after which a portion of the ligation products were transformed into *E. coli*.

12.2. TRANSFORMATION OF *ESCHERICHIA COLI*

Recombinant DNA was transformed into one of two *E. coli* strains depending on the experiment.

PREPARATION OF COMPETENT *E. COLI* TG1

A single colony of *E. coli* TG1 (phage and plasmid free) was inoculated into 50ml of MGT medium and incubated overnight at 37°C with vigorous shaking (~180rpm). Fifty ml of LB broth was inoculated with 200µl of the overnight culture and incubated at 37°C with vigorous shaking (~180rpm) for about 2 hours until very slight turbidity was observed ($OD_{550} \approx 0.3$). The bacteria were harvested by centrifugation at 4500rpm (3100×*g*) in a Heraeus Labofuge 6000 for 15 minutes. The cells were then resuspended in 20ml of cold (4°C), sterile 100mM calcium chloride solution and incubated on ice for 40-60 minutes, before re-pelleting the bacteria at 3100×*g* for 15 minutes at 4°C. The bacterial cell pellet was finally resuspended in 4ml of cold 100mM calcium chloride solution and stored on ice for 2-4 hours prior to use.

TRANSFORMATION OF *E. COLI* TG1

Approximately half of the ligation products solution was mixed with 200µl of competent *E. coli* TG1 and incubated on ice for 40 minutes. The transformation mix was heat shocked at 42°C for 2 minutes, prior to the addition of 800µl of pre-warmed (to 37°C) LB broth. The mix was incubated at 37°C for 55 minutes then plated out onto selective media. For 'Lac (blue-white) selection' of recombinant clones, 50µl of 2%W/v of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) in *N*,*N*-dimethylformamide (DMF) and 10µl of 0.24%W/v of isopropyl-β-D-thiogalactopyranoside (IPTG) were added to the transformation mix, immediately prior to plating out onto selective agar.

For the Lac selection of recombinant M13 vectors, 200µl of a culture of *E. coli* TG1 (in the mid-log phase of cell growth), 50µl of 2%W/v X-gal (in DMF), and 10µl of 0.24%W/v IPTG were added to the transformation mix after heat shock, in place of the 800µl of LB broth described above. Molten LB top agar (2ml cooled to ~55°C) was mixed with the transformed cells and poured evenly over the surface of a pre-warmed (37°C) LB agar plate. The top agar

was left to solidify at room temperature, before incubation of the plates at 37°C overnight. Plaques of transfected *E. coli* TG1 were 'picked' with a sterile glass Pasteur pipette by removing an agar plug containing the entire plaque, which was then transferred to a suitable growth medium.

TRANSFORMATION OF *E. COLI* DH5α

MAX Efficiency DH5α Competent Cells (Gibco BRL) were used for the transformation of recombinant plasmids containing PCR-amplified DNA. The MAX Efficiency DH5α Competent Cells were prepared by Gibco BRL from *E. coli* DH5α with a modification of the procedure described by Hanahan [169]. The transformation procedure recommended by the manufacturer was followed.

13. SITE-DIRECTED MUTAGENESIS

The 'Oligonucleotide-directed *in vitro* mutagenesis system (Version 2.1)' (Amersham International plc) was used for the introduction of point mutations into the *bla*T-1 gene. The system is based on the findings of Eckstein and co-workers [229-232]. Figure 2.3 (page 81) summarises the manufacturer's recommended protocol [168].

All oligonucleotide primers for SDM were supplied by Glaxo Group Research Ltd., and the specific sequences of the primers are given under the relevant section in Chapter 3. For each SDM experiment an oligonucleotide primer, with a single mismatched nucleotide to the target sequence, was annealed to the single-stranded M13 DNA template (see Section 9.4 page 68 above for preparation of templates). Heteroduplex DNA was then formed by polymerisation of a DNA strand with Klenow fragment and T4 DNA ligase. The newly synthesised DNA strand contained phosphorothioate, and was thus protected from *Nci* I digestion [229, 231]. The non-mutant DNA strand was 'nicked' with *Nci* I (recognition site ~500-bp from the M13 multiple-cloning site) and degraded in the 3'→5' direction from free 3'-DNA ends with the exonuclease *Exo* III [232]. Re-synthesis of the double-stranded closed-circular DNA ensured homoduplex formation, hence each strand encoded the point mutation required. The mutant homoduplex DNA was used for the transformation of *E. coli* TG1 as described above (Section 12.2 page 79).

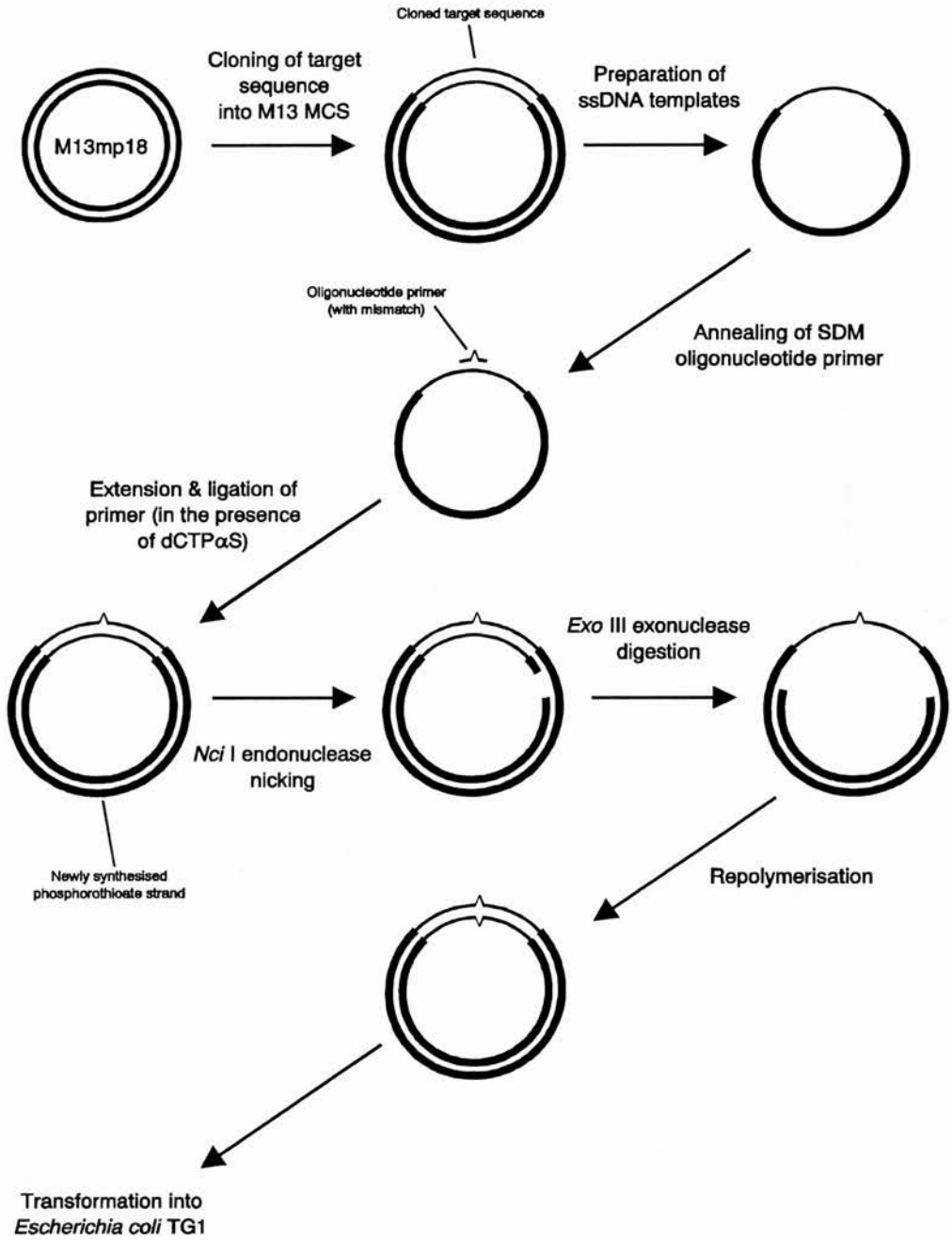

Figure 2.3: Summary of the Amersham oligonucleotide-directed *in vitro* mutagenesis system

Abbreviations: MCS, multiple cloning site; SDM, site-directed mutagenesis; ssDNA, single-stranded DNA. After Amersham International plc [168].

Chapter 3

RESULTS

1. PROLOGUE

The results of the research executed for the completion of this work has been divided into seven sections. The first section describes the identification and characterisation of ten clinical isolates of 3GC resistant *Klebsiella* from a London hospital (the results of which have been published [232]). These isolates contained an example of the ESBL mutations that are the subject of this thesis, and are a reminder of the clinical relevance of the need to identify the mechanisms of ESBL mutation and why such mutations have not become ubiquitous. In essence, the ESBL mutations do not appear to be self-perpetuating; but appear to be 'unstable' in nature, according to the relative rarity of published reports.

The subsequent sections describe the design of a classification scheme for the TEM- and SHV-*derived* β-lactamases and how this scheme was used to investigate the relationships of the TEM-*derived* β-lactamases. The elucidation of the DNA sequence of the first known ESBL, TEM-E2, is described; and the nature of the ESBL mutation holds crucial significance in the development of the ESBL during the 1980s and 1990s. Finally, the effect of the selective pressure of two antimicrobial agents were investigated.

2. IDENTIFICATION OF β-LACTAMASES FROM CLINICAL ISOLATES

Ten clinical isolates of *Klebsiella pneumoniae* were received from the Department of Medical Microbiology, Royal Free Hospital, Pond Street, London NW3 2QG in Autumn 1990. Identification of the resistance mechanisms of the strains, sequentially numbered KR1 to KR10 (for '*Klebsiella* Resistant'), was carried out. The origin of the ten strains was not known until identification of the β-lactamases present in each had been completed. The 'blind' nature of the identification was not the intention of either Dr MacDonald or myself, but was introduced by other parties for an unknown reason. The lack of information regarding the origin of isolation of the strains influenced the methodology of study.

Resistance phenotypes were initially determined by measuring the zone of inhibition of antibiotic discs for ampicillin, ceftazidime, and cefotaxime. Crude extracts of each of the ten strains were prepared and the pI of any β-lactamase produced was determined by IEF. Crude cell free β-lactamase extracts were then used to determine the specific activity of each enzyme preparation, and compared with standard β-lactamase preparations.

2.1. CASE HISTORIES

The ten strains were isolated from three patients, eight from Case 1 (KR1→KR8), and one each from Case 2 (KR9) and Case 3 (KR10). The case history of each patient was supplied by Dr MacDonald and each is summarised below.

CASE 1: A 17 year old man received chemotherapy of Acute Myeloid Leukaemia in Turkey after diagnosis in mid-1989, and remission was achieved. In late-1989 he was admitted to the Royal Free Hospital (RFH) for further chemotherapy, in preparation for bone marrow transplantation (BMT). Whilst in hospital the patient, suffering from neutropenia, experienced two separate episodes of pyrexia. Four antibiotics (colistin, ciprofloxacin, aztreonam and azlocillin) were administered at this time: the two former agents for prophylaxis and the latter two agents for treatment of the pyrexia. A ciprofloxacin and trimethoprim resistant *Kleb. pneumoniae* was isolated from surveillance culture of stools. Prior to re-admission to the RFH, for the proposed BMT in Spring

1990, the patient received a short course of pivampicillin for the treatment of an infected ingrown toenail.

Figure 3.1 represents the drug treatment administered to the patient during re-admission to the RFH for the BMT. The day of isolation for each of the eight *Klebsiella* strains (KR1→KR8) is indicated in the diagram. Ten days after the BMT, and whilst still neutropenic, the patient developed a pyrexia and was treated with amikacin and azlocillin. *Kleb. pneumoniae* were isolated from blood cultures, but not from sputum, urine or stools, on four separate occasions during this episode (Figure 3.1). The patient was then treated with low dose imipenem (500mg i.v. qds) to which the fever initially responded. Pyrexia had developed again after four days and a final *Kleb. pneumoniae* was isolated from the blood three days later. Increasing the dose of imipenem (1g i.v. qds) abolished fever. Imipenem was given orally on return of a normal neutrophil

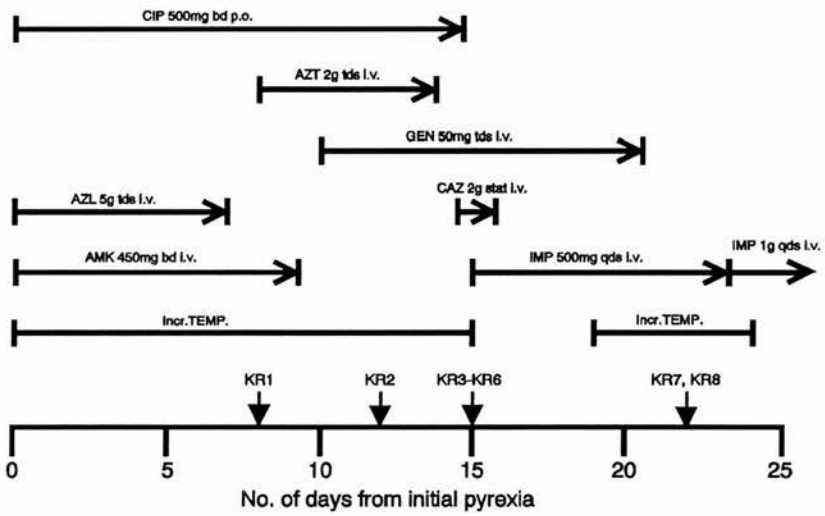

Figure 3.1: Diagram representing the case history of Case 1 from the Royal Free Hospital.

Scale in days from start of initial pyrexia experienced during the second admission to the RFH. Abbreviations: AMK, amikacin; AZL, azlocillin; AZT, aztreonam; bd, twice daily; CAZ, ceftazidime; CIP, ciprofloxacin; GEN, gentamicin; i.v., intravenous infusion; IMP, imipenem; Incr. TEMP., periods of pyrexia; qds, four times daily; p.o., oral administration; stat, single dose; tds, three times daily. Strains of *Klebsiella pneumoniae* KR1 to KR8 were isolated from blood cultures taken on the days indicated.

count, but the patient became pyrexial again for two days after termination of antibiotic treatment. The fever responded on re-instigation of oral imipenem therapy. Imipenem was given for a total of 60 days during this prolonged episode of intermittent *Kleb. pneumoniae* septicaemia.

CASE 2: A previously healthy 16 year old girl developed Guillamune-Barré Syndrome after an upper respiratory tract infection. The girl was treated for four weeks in an Athens ITU where she developed a large sacral sore. Two weeks later the patient was transferred to the RFH, and was admitted on the same day as the above patient (Case 1) for treatment of the sore and convalescence. On arrival her medications, which included imipenem, were stopped. Urine specimens, taken soon after admission, grew multiply resistant *Kleb. pneumoniae*. The patient remained well during the two months of hospital treatment, required no antibiotic therapy and was discharged following a successful skin graft. The patient was treated in isolation because of continued urinary colonisation of the *Klebsiella*. Strain number KR9 was taken as a representative strain of the multiply resistant *Kleb. pneumoniae* isolated from the urine of this patient.

CASE 3: A 28 year old paraplegic man (since a C5 crush fracture of the cervical spine in 1985) was admitted to the RFH with urinary retention and fever in mid-1990. The indwelling electronic device controlling the ureteric and anal sphincters had malfunctioned. *Kleb. pneumoniae* were isolated from both blood and urine specimens, and the infection did not respond to 24 hours of cefotaxime treatment. The patient was subsequently successfully treated with amikacin followed by oral trimethoprim. The patient was nursed in isolation until three consecutive urine specimens were found to be clear of the organism. Since 1985 this patient had been an inpatient at two other London hospitals and had no history of foreign travel. Strain number KR10 was representative of the initial isolates of *Kleb. pneumoniae* from blood and urine.

2.2. MICs FOR THE CLINICAL STRAINS KR1 TO KR10

The resistance phenotype for each of the ten strains was determined by measurement of zone of inhibition of growth of ampicillin, ceftazidime and

cefotaxime sensitivity test discs. IST agar plates were flood seeded with overnight cultures of the organism under test. The results, given in Table 3.1, followed the trend of the results of the MIC determinations for the ten strains provided by Dr Alan MacDonald (RFH) (see Table 3.2 page 87). Dr MacDonald determined the MICs of the ten clinical isolates for a range of β-lactam antimicrobial agents alone, and in combination with clavulanic acid (2mg/l), at two inoculation concentrations (10^4cfu/spot and 10^6cfu/spot) (Table 3.2 page 87).

Strain number	Zone of inhibition (Ø mm)‡			Phenotype§		
	AMP	CAZ	CTX	AMP	CAZ	CTX
KR1	Nil	20	21	R	S	S
KR2	Nil	26	36	R	S	S
KR3	Nil	13	15	R	I	S
KR4	Nil	10	19	R	R	S
KR5	Nil	12	22	R	I	S
KR6	Nil	14	13	R	R	I
KR7	Nil	30	33	R	S	S
KR8	Nil	23	19	R	S	S
KR9	Nil	7	13	R	R	R
KR10	Nil	Nil	21	R	R	S
Controls†:						
OXA-2	12	27	36	I	S	S
TEM-1	Nil	30	35	R	S	S

Table 3.1: Antibiotic sensitivity of clinical isolates of ceftazidime resistant *Klebsiella pneumoniae* from the Royal Free Hospital.

Antibiotic sensitivity testing with antibiotic sensitivity discs following the BSAC guidelines [196]. Abbreviations: Ø, diameter; AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; Nil, no zone of inhibition of growth. ‡ Zone diameters given in mm (disc diameter 6mm) for the following disc concentrations: 25μg ampicillin/disc, 30μg ceftazidime/disc, 30μg cefotaxime/disc. § Phenotype: I, intermediate (zone Ø > 10mm, < 14mm); R, resistant (zone Ø ≤ 10mm); S, sensitive (zone Ø ≥ 14mm). † *E. coli* J62-2 expressing the given β-lactamase.

Anti-biotic	MIC (mg/l) for strain no.									
	KR1	KR2	KR3	KR4	KR5	KR7	KR8	KR9	KR10	NCTC
CAZ	0.5	4	32	128	128	64	32	64	512	0.125
	0.5	16	128	512	512	512	64	512	>1024	0.125
CAZ/ Clav	0.25	0.5	1	1	1	64	8	0.25	4	0.125
	0.5	4	4	8	8	128	32	0.5	4	0.125
CTX	0.25	2	16	4	4	256	64	2	16	0.015
	0.5	256	512	256	256	>1024	1024	32	64	0.25
CTX/ Clav	0.25	0.25	0.25	0.25	0.25	128	16	0.063	2	0.016
	0.5	8	0.5	0.5	0.5	256	128	0.125	0.125	0.25
AZT	0.125	2	16	128	128	128	16	64	64	0.063
	0.25	16	64	512	512	128	64	512	>512	0.063
AZT/ Clav	0.125	0.125	0.125	0.125	0.125	16	4	0.063	1	0.063
	0.125	4	0.25	4	4	128	32	0.125	0.25	0.063
IMP	0.125	0.125	0.5	0.5	0.25	2	2	0.063	1	0.25
	0.5	1	0.5	1	1	>2	>2	0.5	1	0.5
IMP/ Clav	0.125	0.063	0.125	0.125	0.125	1	1	0.063	0.5	0.125
	0.5	1	1	0.5	0.5	2	1	0.5	1	0.5
TEM	8	8	8	8	8	32	16	4	16	2
	8	8	16	16	16	64	16	8	16	4
TEM/ Clav	4	4	4	4	4	16	16	4	8	1
	8	8	8	8	8	32	32	4	8	4
CEF	16	8	8	8	8	128	64	8	64	4
	32	64	16	16	16	128	64	16	8	4
CEF/ Clav	16	8	8	8	8	128	64	64	4	4
	32	64	64	32	16	128	128	8	32	4

Table 3.2: MICs for clinical isolates of ceftazidime resistant *Klebsiella pneumoniae* from the Royal Free Hospital.

MIC results from Dr A. MacDonald, Dept. of Medical Microbiology, Royal Free Hospital, London. All strains incubated overnight (18-24 hours) prior to inoculation. 24 hour aerobic incubation at 37°C following inoculation of isosensitest agar plates containing required antibiotic concentration with 1μl of test organism (10^4cfu/spot and 10^6cfu/spot: upper and lower figures in each row respectively). Abbreviations: AZT, aztreonam; CAZ, ceftazidime; CEF, cefoxitin; Clav, clavulanic acid (2mg/l) ; CTX, cefotaxime; IMP, imipenem; NCTC, *E. coli* NCTC 10418; TEM, temocillin.

2.3. ANALYTICAL ISOELECTRIC FOCUSING

Crude β-lactamase extracts of the ten clinical strains were applied, as well as extracts of bacteria with known and well characterised enzymes, to analytical IEF gels in order to identify the isoelectric point of the unknown enzymes. IEF provided the following results: nine of the isolates (KR2→KR10) exhibited β-lactamase bands at pI 5.4, identical to the pI of TEM-1 (Figure 3.2 page 89). All isolates contained a pI ~7.0 enzyme band (a similar value to many SHV-*type* and chromosomal enzymes); in seven of these isolates this enzyme band was faint. The pI ~7.0 band was strong in three preparations: KR1, KR9 and KR10. One strain (KR10) contained a β-lactamase with pI 5.57 similar to that of TEM-E3 and TEM-10 (see Figure 3.2 page 89). Nine of the isolates contained two enzymes, one isolate (KR1) only exhibited a single enzyme (pI ~7.0).

2.4. RELATIVE RATES OF HYDROLYSIS

Relative rates of hydrolysis of the β-lactamases from a selection of the ten *Kleb. pneumoniae*, together with a range of known β-lactamases were determined for comparison. The results of this analysis are summarised in Table 3.3 (page 90) where the values are normalised for comparison with ampicillin (100%). These results show that the unknown enzyme of pI 5.57, from *Kleb. pneumoniae* KR10, is very similar in hydrolytic characteristics to TEM-10 or TEM-E3 (the latter enzyme is thought to be an analogue of TEM-10 [192]).

2.5. TRANSCONJUGATION & TRANSFER OF CEFTAZIDIME RESISTANCE

The *Kleb. pneumoniae* isolate producing the unidentified pI 5.57 enzyme was conjugated with *E. coli* J62-2 (Rif^R *his lac pro trp*) and transconjugants counter-selected with rifampicin, ampicillin and ceftazidime. Following IEF, two transconjugants were shown to produce the pI 5.57, one with and one without the pI 5.4 enzyme. The pI ~7.0 enzyme was not found to be present in any recipient strains after transconjugation (Figure 3.3 page 91).

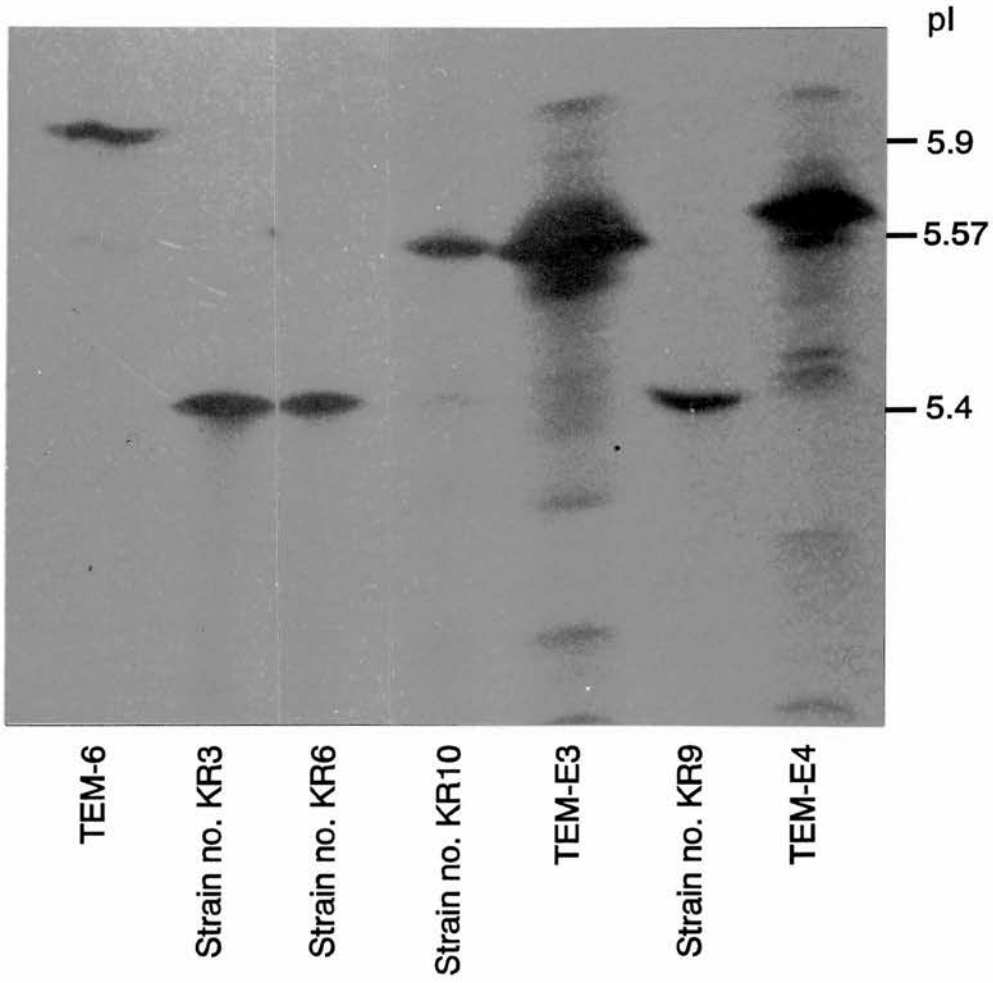

Figure 3.2: IEF gel showing the TEM-*type* β-lactamases of *Klebsiella pneumoniae* KR3, KR6, KR9 & KR10 from the Royal Free Hospital

IEF gel contained carrier ampholytes pH range 4-6. Unknown enzymes shown were from crude extracts of the corresponding clinical strains described in the text. Standard β-lactamases shown include TEM-E3 (pI 5.57), TEM-E4 (pI 5.61), and TEM-6 (pI 5.9).

Strain	pI	Relative rates of hydrolysis (%) §					
		AMP	NCFN	CFX	CEP	CAZ	CTX
KR1	~7.0	100	118	3.2	47.1	0.1	3.5
KR2	5.4, (~7.0)						
KR3	5.4, (~7.0)	100	0.5			0.02	
KR4	5.4, (~7.0)						
KR5	5.4, (~7.0)						
KR6	5.4, (~7.0)	100	5.7			0.03	
KR7	5.4. (~7.0)						
KR8	5.4, (~7.0)						
KR9	5.4, ~7.0	100	33.1			2.3	
KR10	5.4, 5.57, ~7.0	100	43.9	2.1	88.5	17.5	2.1
TEM-1	5.4	100	18.2			UM	UM
OXA-2	7.4	100	73.3			0.1	
TEM-5	5.5	100		52.5	425.5	35.1	128.3
TEM-10	5.57	100		3.8	67.9	45.8	1.7
TEM-12	5.25	100	4.4			0.01	0.01
TEM-E3	5.57	100		3.0	26.7	218	0.1
J62-2	-	UM	UM	UM	UM	UM	UM
Strain A‡	5.57	100		8.2	181	56.5	8.2
Strain B‡	5.57, (5.4)	100		3.6	49.6	21.2	1.2

Table 3.3: Hydrolytic characteristics of clinical isolates of ceftazidime resistant *Klebsiella pneumoniae* from the Royal Free Hospital.

§ Relative rate of hydrolysis (%) with respect to ampicillin (100%). ‡ Two ceftazidime resistant *E. coli* J62-2 transconjugants (Strain A & Strain B) of *Kleb. pneumoniae* 1604 (KR10). Abbreviations: AMP, ampicillin; CAZ, ceftazidime; CEP, cephaloridine; CFX, cefuroxime; CTX; cefotaxime; NCFN, nitrocefin; pI, isoelectric point; UM, un-measurable (rate of hydrolysis very slow or non-existent).

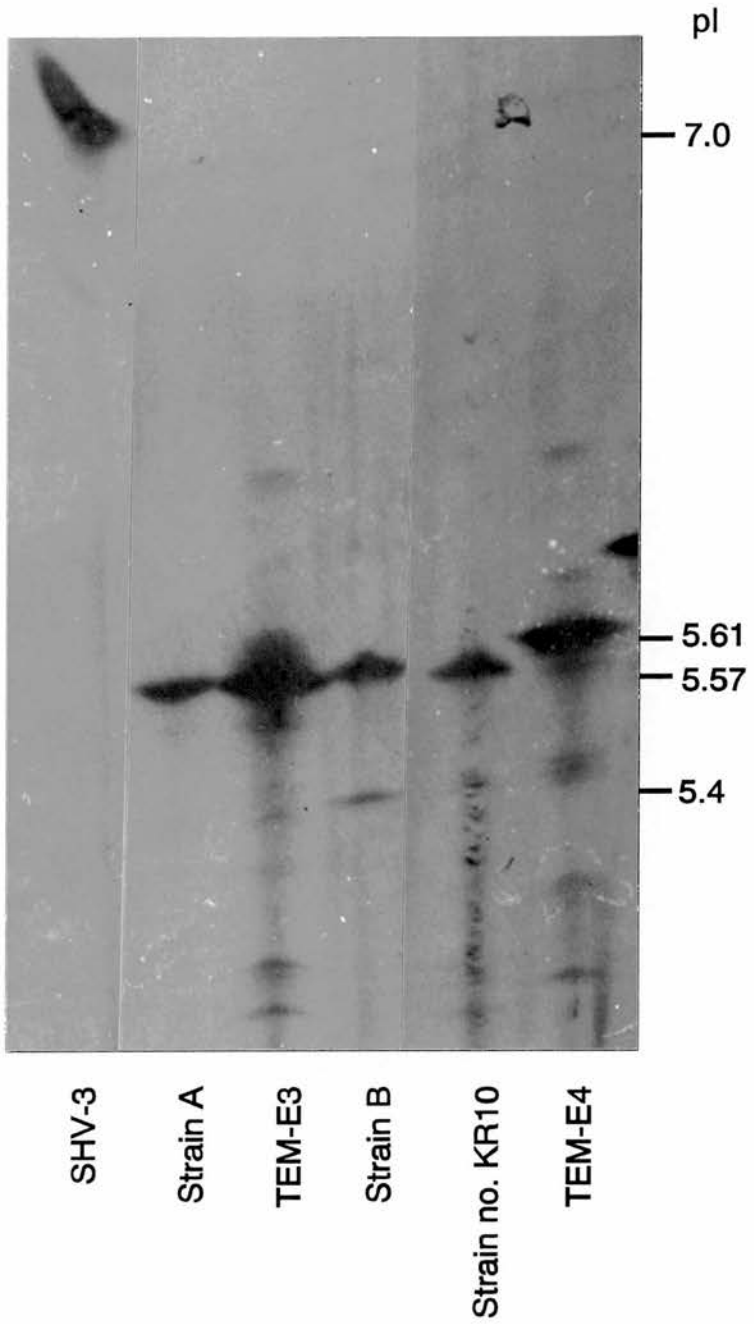

Figure 3.3: IEF gel showing the TEM-*type* β-lactamases of *Klebsiella pneumoniae* KR10 from the Royal Free Hospital & transconjugants

IEF gel contained mixture of carrier ampholytes pH ranges 3.5-10 and 4-6. Standard β-lactamases shown include SHV-3 (pI 7.0), TEM-E3 (pI 5.57), & TEM-E4 (pI 5.61). β-Lactamases labelled Strain A and Strain B are from two *E. coli* J62-2 transconjugants of *Kleb. pneumoniae* KR10 (see text for details).

2.6. GEL ELECTROPHORESIS OF PLASMID DNA

Previous work by Payne [141, 192] gave rise to the hypothesis that TEM-E3 and TEM-10 may be the same enzyme. Electrophoresis of intact plasmid DNA and plasmid DNA digested with *Eco*R I and *Sca* I was performed to elucidate similarities between two TEM-E3 encoding plasmids (pUK722 and pUK723), the TEM-1 plasmid R1, and the plasmid encoding the unknown pI 5.57 enzyme. Results suggested that each enzyme was present on different large plasmids.

2.7 CONCLUDING REMARKS

Analysis of the MIC data (Table 3.2 page 87) showed that multiple-drug resistance of the *Kleb. pneumoniae* isolated from Case 1 increased as a direct result of therapy. Although the patient was receiving a number of β-lactam antimicrobial agents (including a single dose of ceftazidime), the increase in resistance to 3GCs was not the result of β-lactamase acquisition or mutation; the strains isolated after the instigation of aztreonam therapy had, however, acquired a pI 5.4 enzyme. The identity of the pI 5.4 β-lactamase in the relevant isolates was probably the ubiquitous TEM-1; the pI ~7.0/SHV-*type* enzyme, was probably of chromosomal origin. The isolate from Case 2 (KR9) also probably expressed the TEM-1 enzyme. The single isolate from Case 3 (KR10) possessed three β-lactamases: the pI ~7.0 enzyme was a chromosomal SHV-*type* enzyme; the pI 5.57 enzyme was thought to be TEM-E3/TEM-10.

3. MAPPING OF THE EXTENDED-SPECTRUM β-LACTAMASES

It is known, from the nucleotide or amino acid sequences, that many of the TEM- and SHV-*type* enzymes are very closely related. Many of these β-lactamases differ by between one and three amino acid residues from the parental enzymes: TEM-1, TEM-2 or SHV-1. A single exception, TEM-24, differs from the parental enzyme TEM-2 by four amino acid substitutions (see Chapter 1, Table 1.9 page 45).The amino acid sequences of TEM-1 (pI 5.4) and TEM-2 (pI 5.6) differ at a single residue, amino acid 39 [149, 150]. This amino acid substitution of lysine for glutamine is the result of a single nucleotide change of cytosine to adenine at position 317 (numbering according to Sutcliffe (1978) [150]). All the other amino acid substitutions that have been determined are the result of a single nucleotide change. Referring to Figure 3.4a below: if the parental β-lactamase is represented by Enzyme A a single amino acid substitution (X, represented by the arrow) could create Enzyme B, or another substitution (Y) could create Enzyme C. Enzyme B can also undergo substitution Y to produce a third enzyme, D. The same is true of Enzyme C, with substitution X (see Figure 3.4b).

The series of mutations, therefore, that have created Enzyme D from Enzyme A can occur by two distinct routes: either via Enzyme B or Enzyme C. The number of permutations increase rapidly with the number of mutations that occur. In the latter half of 1990 two maps were introduced [234] that identified all of the possible intermediates of the SHV- and the TEM-*derived* ESBL sequenced at that time.

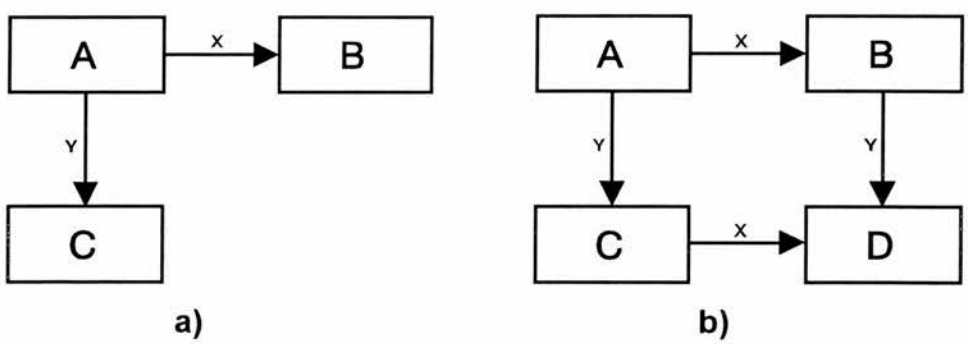

Figure 3.4: Theoretical relationship of β-Lactamases

Each box represents a β-lactamase and each arrow represents a single amino acid substitution.

3.1. THE SHV-*DERIVED* ENZYMES

The primary structures of the SHV-*derived* β-lactamases SHV-1 to SHV-5, and SHV-8 have been determined. SHV-8, an enzyme created *in vitro* by SDM, does not exhibit any significant ESBL activity [235]. Each of these enzymes have been numbered chronologically according to isolation (or creation), rather than denoting the evolutionary distances from SHV-1 (this is the same for the TEM-*derived* enzymes). SHV-2 was the first SHV-*derived* ESBL to be sequenced, and it was shown to differ from SHV-1 by a single amino acid change of glutamic acid at position 238 to serine. All the clinically isolated SHV-1-*derived* ESBL sequenced so far have possessed a serine residue at position 238 rather than a glutamic acid residue. It is therefore, concluded that each of the subsequently identified SHV-*derived* ESBL (SHV-3, -4, and -5) are derived from SHV-2, which was derived from SHV-1; a fact that considerably simplifies the map of SHV-enzyme mutations [234]. Figure 3.5 (page 95) represents the inter-relationship of the SHV-1-*derived* β-lactamases and it is obvious from this map that the ultimate ESBL of this series, SHV-4, can be derived through mutation from SHV-3 or SHV-5. The map of the inter-relationships of the clinically isolated SHV-*derived* ESBL (Figure 3.5) has changed little since its introduction [234] in 1990. Other SHV-*derived* enzymes have been identified (e.g. SHV-6 [236]), but details of the amino acid sequences of the enzymes have not appeared in primary journals.

3.2. THE TEM-*DERIVED* ENZYMES

The inter-relationship of the TEM-*derived* β-lactamases, because of the number of enzymes that have been identified and sequenced, is more complicated than the inter-relationships of the SHV-1-*derived* enzymes. At the time the map defining the TEM-*derived* inter-relationship was introduced [234], there were 17 possible intermediate enzymes between the eight sequenced TEM-*type* ESBL and the two parental enzymes (TEM-1 and TEM-2). Many of the intermediates in the intervening three years have been identified clinically. Determination of the amino acid sequences of many of the TEM-*type* β-lactamases have filled the gaps in the map. Table 3.4 (page 97) shows the amino acid substitutions between each of the 21 sequenced TEM-β-lactamases, and the percentage of amino acid identity between each of the mature β-lactamases.

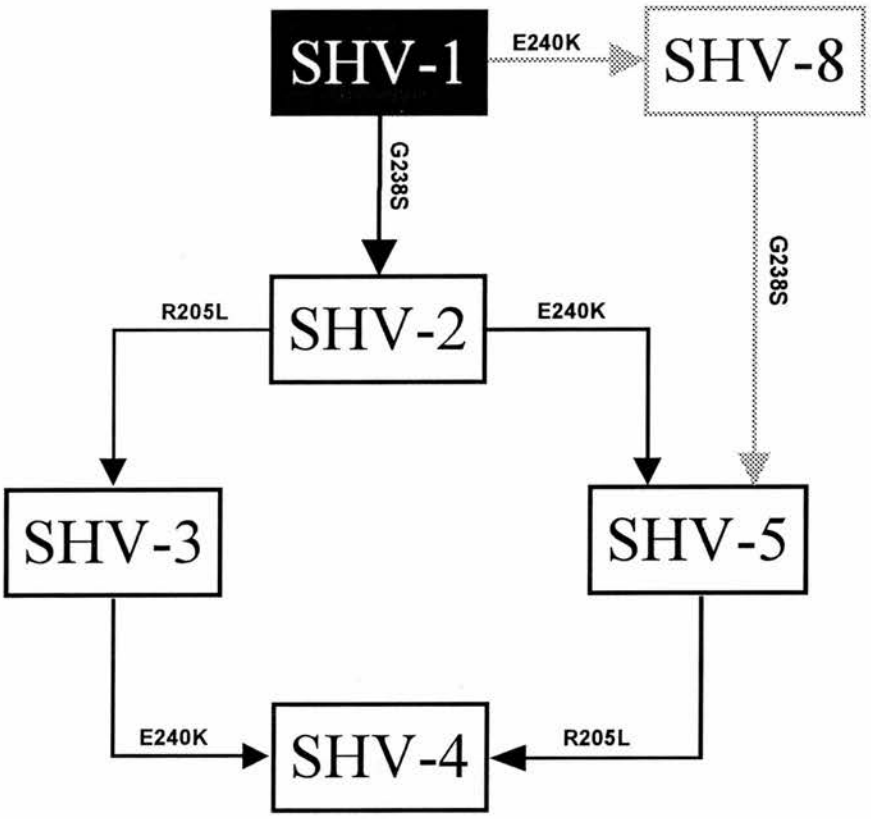

Figure 3.5: Representation of the inter-relationship of the SHV-*derived* β-lactamases

Each arrow represents a single amino acid change facilitated by a single nucleotide change in the β-lactamase gene. SHV-8 has not been identified clinically, hence, is shown shaded. One-letter amino acid notation according to the IUPAC-IUB Commission on Nomenclature [237]. Numbering of the amino acid residues is according to the recommendations of Ambler *et al* (1991) [158], and places the active-site serine residue at amino acid position 70.

Figure 3.6 (page 98) is the revised representation with all sequenced enzymes included. Apart from one notable exception (TEM-24) all the end-point enzymes can be connected to the parental enzymes TEM-1 and TEM-2 by a series of clinically isolated enzymes that only differ in primary structure by a single amino acid substitution. The missing intermediates, between TEM-7 and TEM-24, have been called TEM-X and TEM-Y for the purposes of this thesis. TEM-X is also possible predecessor of TEM-8, which is otherwise thought to have been derived from TEM-2 via TEM-18 and TEM-3.

The mutation between TEM-1 and TEM-2 occurred, it can probably be assumed, before later enzymes emerged, therefore, the change from glutamine to lysine at position 39 does not occur within the ESBL. Two clearly separate TEM-*derived* enzyme groups, therefore, exist in the TEM-derived β-lactamases: TEM-1-*derived* and TEM-2-*derived*. The amino acid substitution table below (Table 3.4 page 97) does not take account of the TEM-1/TEM-2 division. The final column of the table gives the mean number of substitutions between any enzyme and the other 20 β-lactamases listed. The higher the value the more peripheral the enzyme, and conversely, the lower values indicate the central enzymes. TEM-1, TEM-2, TEM-17, and TEM-18 are the most 'central', and TEM-5 and TEM-24 are the most 'peripheral'. Under this assumption it is interesting to note that all but a single ESBL (TEM-24) of the SHV-*derived*, TEM-1-*derived* or TEM-2-*derived* enzyme groups is no more than three amino acid substitutions distant from its respective parental β-lactamase. It has become apparent from this representation that some of the intermediate enzymes are common predecessors for more than one end-point ESBL, hence could be considered the more 'popular' mutations.

		Number of amino acid substitutions																					
	TEM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	24	26	$\bar{x}$
P	1		1	3	3	3	2	2	4	3	2	2	1	2	4	2	3	1	2	1	5	2	2.5
e	2	0.4		2	4	4	3	1	3	4	3	1	2	1	3	3	2	2	1	2	4	3	2.5
r	3	1.1	0.4		2	6	3	3	1	4	5	3	4	3	1	1	2	2	1	2	4	3	2.8
c	4	1.1	1.5	0.8		6	2	5	3	2	5	5	4	3	1	1	4	2	3	2	6	3	3.4
e	5	1.1	1.5	2.3	2.3		5	3	5	4	1	5	2	5	7	5	6	4	5	4	2	3	4.3
n	6	0.8	1.1	1.1	0.8	1.9		4	4	3	4	2	3	4	4	2	1	1	2	3	5	2	3.0
t	7	0.8	0.4	1.1	1.9	1.1	1.5		2	3	2	2	1	2	4	4	3	3	2	3	3	2	2.8
a	8	1.1	1.5	0.4	1.1	1.9	1.5	0.8		3	5	4	3	4	2	2	3	3	2	3	3	2	3.1
g	9	1.1	1.5	1.5	0.8	1.5	1.1	1.1	1.1		3	5	2	3	3	3	4	2	3	4	4	1	3.2
e	10	0.8	1.1	1.9	1.9	0.4	1.5	0.8	1.9	1.1		4	1	4	7	4	5	3	4	3	3	2	3.6
	11	0.8	0.4	1.1	1.9	1.9	0.8	0.8	1.5	1.9	1.5		3	2	4	4	1	3	2	3	5	4	3.3
d	12	0.4	0.8	1.5	1.5	0.8	1.1	0.4	1.1	0.8	0.4	1.1		3	5	3	4	2	3	2	4	2	2.8
i	13	0.8	0.4	1.1	1.1	1.9	1.5	0.8	1.5	1.1	1.5	0.8	1.1		2	4	3	3	2	3	5	4	3.2
f	14	1.5	1.1	0.4	0.4	2.7	1.5	1.5	0.8	1.1	2.7	1.5	1.9	0.8		2	3	3	2	3	5	4	3.5
f	15	0.8	1.1	0.4	0.4	1.9	0.8	1.5	0.8	1.1	1.5	1.5	1.1	1.5	0.8		3	1	2	1	5	2	2.8
e	16	1.1	0.8	0.8	1.5	2.3	0.4	1.1	1.1	1.5	1.9	0.4	1.5	1.1	1.1	1.1		2	1	4	4	3	3.1
r	17	0.4	0.8	0.8	0.8	1.5	0.4	1.1	1.1	0.8	1.1	1.1	0.8	1.1	1.1	0.4	0.8		1	2	4	1	2.3
e	18	0.8	0.4	0.4	1.1	1.9	0.8	0.8	0.8	1.1	1.5	0.8	1.1	0.8	0.8	0.8	0.4	0.4		3	3	2	2.4
n	19	0.4	0.8	0.8	0.8	1.5	1.1	1.1	1.1	1.5	1.1	1.5	0.8	1.1	1.1	0.4	1.5	0.8	1.1		6	3	2.9
c	24	1.9	1.5	1.5	2.3	0.8	1.9	1.1	1.1	1.5	1.1	1.9	1.5	1.9	1.9	1.9	1.5	1.5	1.1	2.3		3	4.2
e	26	0.8	1.1	1.1	1.1	1.1	0.8	0.8	0.8	0.4	0.8	0.8	0.8	1.5	1.5	0.8	1.1	0.4	0.8	1.1	1.1		2.6

Table 3.4: The inter-relationship of the TEM-*derived* β-lactamases

Number of amino acid changes between each TEM-*derived* β-lactamases (top right) and percentage of amino acid sequence difference between β-lactamases (bottom left). The right hand column, $\bar{x}$, gives the mean amino acid substitution distance between the mature-enzyme (263 amino acids) and the other TEM-*derived* enzymes. Other variations have been observed in the leader sequence of the precursor-β-lactamase (286 amino acid residues) but are not included.

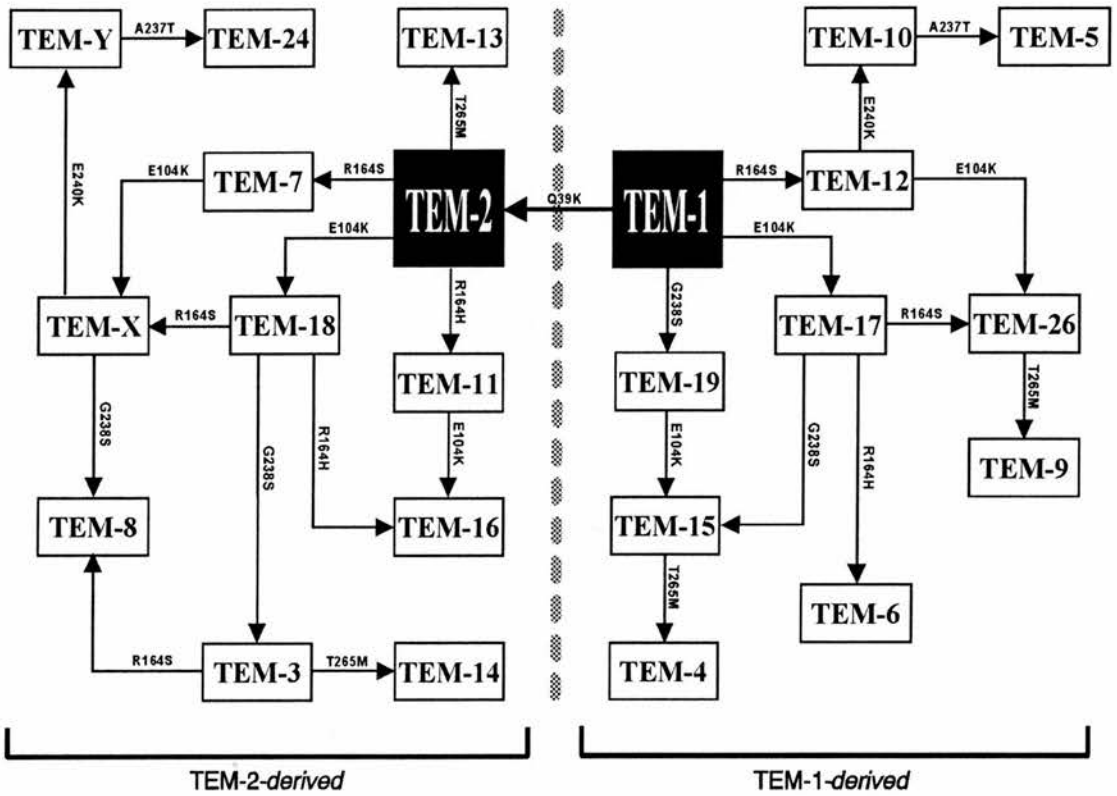

Figure 3.6: Representation of the inter-relationship of the TEM-*derived* β-lactamases

Each arrow represents a single amino acid change, facilitated by a single nucleotide change, in the β-lactamase gene. Enzymes are divided into two groups: TEM-1-*derived* and TEM-2-*derived* along the Q39K change between TEM-1 and TEM-2. The enzymes named TEM-X and TEM-Y in this diagram have not been found clinically (see text for details: Section 3.2 page 96). One-letter amino acid notation according to the IUPAC-IUB Commission on Nomenclature (1969) [237]. Numbering of the amino acid residues is according to the recommendations of Ambler *et al* (1991) [158].

4. GENERATION OF TEM-*DERIVED* β-LACTAMASES BY SITE-DIRECTED MUTAGENESIS

After the first version of the map representing the TEM-*derived* β-lactamases was drawn in Autumn 1990 [234] very few of the intermediate ESBL had been sequenced and/or were available for study. It was proposed that some of the intermediate enzymes between TEM-1 and TEM-5 were to be constructed by site-directed mutagenesis (SDM). The filamentous bacteriophage M13mp18 was chosen as the cloning vector because of the following reasons:

- Ease with which single-stranded sequencing quality DNA could be isolated from cell culture for the verification of mutagenesis of the cloned fragment.
- The range of restriction sites within the multiple cloning site (MCS) of the *lacZ'* gene.
- Lac (or Blue-white) selection of a suitable *E. coli* host containing DNA fragments cloned into the MCS (disrupting the β-galactosidase activity).

The cloning vector pAT153 [170], a 3.7-kb high copy number derivative of pBR322 (4.4-kb), was also chosen as the donor of the β-lactamase gene fragment, and recipient for the modified fragment, because of the following reasons:

- Most, if not all, of the commercially available plasmid cloning vectors that confer ampicillin resistance (Amp^R) as a selectable genetic marker carry the TEM-1 β-lactamase gene, *bla*T-1.
- pAT153 has had the *rop* gene present in pBR322 deleted, increasing the copy number compared to the parent vector.
- Copy number can be increased from 20 to 1000 copies per chromosome per bacterium by cultivating the bacteria in the presence of chloramphenicol.
- pAT153 is non-mobilisable because of the deletion of the *bom* site, constituting a further increase in safety compared to pBR322.

4.1. CONSTRUCTION OF AN M13mp18 SDM-VECTOR.

pAT153 and M13mp18 DNA were restricted separately with *Eco*R I and *Pst* I. The resulting DNA fragments were separated by agarose gel electrophoresis, and the 750-bp fragment of pAT153 and the large fragment (~7.2-kb) of M13mp18 were excised and purified by adsorbtion to glass particles (Geneclean II Kit, Bio 101). The purified M13mp18 and pAT153 fragments were annealed and ligated overnight and then used to transform competent *E. coli* TG1. Colonies of *E. coli* harbouring the recombinant M13mp18-750-bp fragment were selected by Lac selection of plaques from a lawn of pure *E. coli* TG1. Mini-prep. DNA was prepared from the selected strains and restricted with *Sca* I (a restriction site present within the 750-bp pAT153 DNA fragment but not found within M13 DNA). Restricted and intact DNA was then analysed by agarose gel electrophoresis. Strains that harboured DNA of the correct size (~8-kb) and that linearised when restricted with *Sca* I were selected for preparation of single-stranded DNA templates for SDM. Orientation of the 750-bp *Eco*R I-*Pst* I DNA fragment from pAT153 in the M13 phage DNA meant that single-stranded templates contained sense sequences, therefore SDM oligonucleotides and sequencing primers were non-sense sequences.

4.2. CONSTRUCTION OF THE RECIPIENT PLASMID VECTOR pUK3001

A plasmid vector, pUK3001 (Figure 3.7 page 101), was constructed in preparation for the cloning of the mutant 750-bp fragment after SDM. pAT153 DNA was restricted with *Eco*R I and *Pst* I, and after purification, was annealed and ligated overnight with a specially designed *Eco*R I-*Xba* I-*Pst* I polylinker fragment (Figure 3.8 page 101). Ligated DNA was then used to transform competent *E. coli* TG1. Bacterial colonies containing plasmid DNA were selected with tetracycline. Colonies that exhibited the tetracycline-resistant and ampicillin-sensitive phenotype were analysed for plasmid DNA (~2.9-kb) that would cut with *Xba* I but not *Sca* I. Bacteria harbouring the recombinant DNA required were used in the purification of pUK3001 with the Qiagen Midi-Prep. Kit (see Chapter 2).

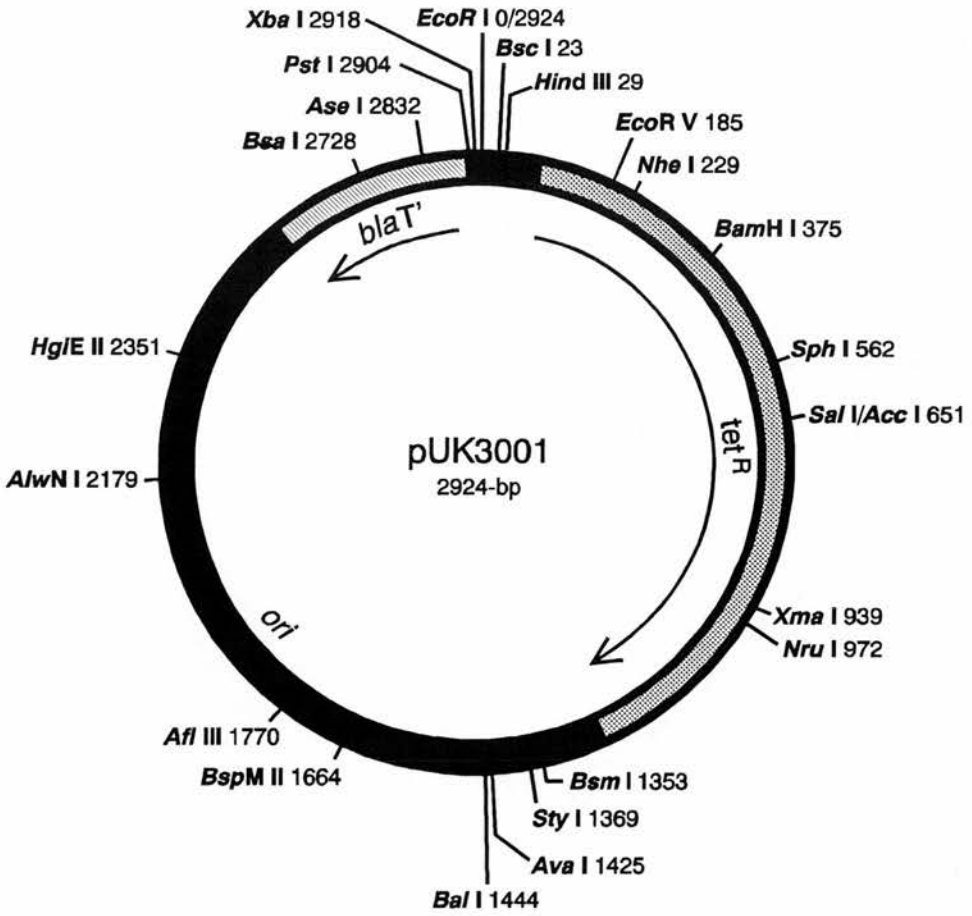

Figure 3.7: Restriction map of pUK3001

The map of the plasmid shows the position of the unique restriction sites for the endonucleases indicated according to various sources (Restriction endonuclease suppliers). Position 0 has been arbitrarily defined as the centre of the *Eco*R I site (nucleotide 1 is the first thymine of the sequence ···GAATTC···). Restriction site positions are given relative to the first base of the recognition sequence (except for *Eco*R I). Abbreviations: *bla*T', remainder of the TEM β-lactamase gene (non-functional but codes for the last 104 amino acids of the enzyme); *ori*, origin of replication; tet^R, tetracycline resistance gene.

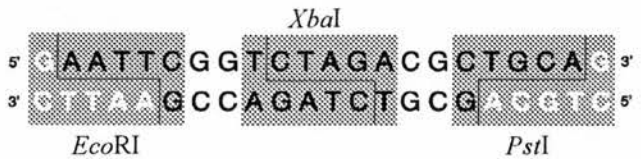

Figure 3.8: *Eco*R I-*Xba* I-*Pst* I polylinker

Polylinker constructed from annealing two oligonucleotides (a 20-mer and a 12-mer) illustrated in black type. The restriction endonuclease recognition sites highlighted by the shaded regions and sites of cleavage are shown. White lettering represents nucleotide bases of recognition sequences not present in the polylinker.

4.3. SDM OF M13mp18-750-bp FRAGMENT VECTOR

SDM of single-stranded M13mp18-750-bp fragment DNA was performed using the 'Oligonucleotide-directed *in vitro* mutagenesis kit' from Amersham International plc [168]. The oligonucleotides listed in Table 3.5 (page 103) were used in SDM experiments

After conducting experiments according to the SDM protocol supplied with the kit for each oligonucleotide, *E. coli* TG1 was transformed with the double-stranded mutant M13mp18-fragment DNA. Plaques were selected from a lawn of uninfected *E. coli* TG1 and grown overnight in LB broth. The overnight cultures were then applied to a GeneScreen Hybridisation Membrane (NEN, DuPont) with the Schleicher & Schuell Minifold II dot-blot apparatus. Negative controls of unmutated single-stranded M13mp18-fragment DNA were used. After denaturing of the DNA and neutralisation of the denaturing solutions, DNA was cross-linked to the hybridisation membrane with UV irradiation. Hybridisation filters were pre-hybridised and then probed with the respective ^{32}P-labelled SDM oligonucleotides. Filters were washed with increasing stringency from a 2×SSC/0.1%W/v SDS wash solution at 37°C to a 0.5×SSC/0.1%W/v SDS wash solution at 50°C until the negative controls gave no appreciable counts above background with a Gieger-Müller tube. Autoradiography of filters revealed possible mutant templates

4.4. SEQUENCING OF M13mp18-750-bp FRAGMENT DNA SUBJECTED TO SDM

Likely mutant M13 templates were used to prepare single-stranded DNA templates for DNA sequencing. The SDM oligonucleotides (Table 3.5 page 103) and the sequencing oligonucleotides listed in Table 3.6 (page 103) were used as primers for sequencing reactions. The whole of the 750-bp fragments, within the M13 vector, that gave positive probing results were sequenced. Table 3.7 (page 104) lists the results of the SDM experiments, all SDM were attempted on the M13 *bla*T-1 template except one which used the template from the Q39K mutagenesis (analogous to *bla*T-2). Each of the M13 templates found to have a desired nucleotide change were sequenced on a minimum of two occasions, to check for mutations that may have been introduced accidentally by the SDM experiment.

Primer‡	Sequence§	Position†	Size	M. Wt
Q39K	5'-GC ACC CAA CT**T** ATC TTC AGC-3'	327-308	20-mer	5997
E104K	5'-GG TGA GTA CT**T** AAC CAA GTC-3'	522-503	20-mer	6141
R164S	5'-CGG TTC CCA AC**T** ATC AAG GC-3'	703-684	20-mer	6062
R164H	5'-CGG TTC CCA A**T**G ATC AAG G-3'	703-685	19-mer	5812
R164D	5'-CGG TTC CCA A**TC** ATC AAG GC-3'	703-684	20-mer	6931

Table 3.5: Oligonucleotide primers for Site-Directed Mutagenesis

‡ Primer nomenclature according to amino acid substitution envisaged. § Oligonucleotides are non-sense strand analogues; nucleotides in bold show mismatches with sequence of *bla*T-1. † 5'→3' binding range of non-sense oligonucleotides to sense strand of *bla*T-1 (DNA sequence numbering according to Sutcliffe (1978) [150]).

Primer‡	Sequence§	Position†	Size	M. Wt
Universal	5'-GTA AAA CGA CGG CCA GT-3'	-	17-mer	-
383(SDM)	5'-GG AAA ACG TTC TTC GGG G-3'	400-383	18-mer	5580
561(SDM)	5'-GGC AGC ACT GCA TAA TTC-3'	578-561	18-mer	5484

Table 3.6: Oligonucleotide primers for DNA Sequencing

‡ Primer nomenclature refers to location of first (5') nucleotide. § Oligonucleotides are non-sense. † 5'→3' binding range of non-sense oligonucleotides to sense strand of *bla*T-1 (DNA sequence numbering according to Sutcliffe (1978) [150]).

Probing identified candidates for DNA sequencing by revealing the SDM-templates exhibiting the required point-mutations. The R164H and R164D SDM experiment either gave false positive probing results (according to the subsequent DNA sequencing of templates), or no positive signals were observed in the various SDM experiments with these oligonucleotides. A summary of the results of probing and DNA sequencing is given in Table 3.7.

4.5. CONSTRUCTION OF MUTANT PLASMIDS pUK3002, pUK3003, pUK3004, & pUK3005

Each mutant template and the recipient plasmid, pUK3001, were restricted with *Eco*R I and *Pst* I. The resulting DNA fragments of the mutant M13 templates were separated by agarose gel electrophoresis, the 750-bp fragments were excised and purified by glass-milk purification. pUK3001 fragments were purified by ethanol precipitation. The mutant fragments were annealed and ligated overnight and the DNA used to transform *E. coli* TG1. Bacterial colonies were selected with

SDM‡	Template§	Probing†	Modifications			Enzyme††
			Nucleotide*	Codon‡‡	Amino acid§§	
Q39K	Q39	+	C-317→A	CAG→AAG	K39	TEM-2
E104K	E104	+	G-512→A	GAG→AAG	K104	TEM-17
R164S	R164	+	C-692→A	CGT→AGT	S164	TEM-12
	K39, R164	+	C-692→A	CGT→AGT	S164	TEM-7
R164H	R164	+(-)	None	-	-	-
R164D	R164	+(-)	None	-	-	-

Table 3.7: SDM Experiments DNA Sequencing Results

‡ SDM oligonucleotide primer used in experiment. § Genotype (amino acid) of M13mp18-750-bp fragment template. † One or more positive signals from oligonucleotide probing of SDM site. * Nucleotide mutations observed in DNA sequence (DNA sequence numbering according to Sutcliffe (1978) [150]). ‡‡ DNA codon modification. §§ Amino acid substitution that would result from the observed nucleotide change. †† Expected recombinant enzyme.

ampicillin and tetracycline.

Restriction analysis of plasmids harboured by Amp^R Tet^R colonies revealed the presence of 3.7-kb plasmids that cut with *Sca* I but not *Xba* I. Re-restriction of recombinant plasmids with *Eco*R I and *Pst* I demonstrated the presence of ~2.9-kb and ~750-bp fragments. The plasmid containing the cytosine-692→adenine mutation was designated pUK3002; the plasmid with the cytosine-317→adenine mutation was designated pUK3003; the plasmid with the guanidine-512→adenine was called pUK3004; the plasmid containing the double mutation of cytosine-317→adenine and cytosine-692→adenine was designated pUK3005. A restriction map of the recombinant plasmids, based on that of pAT153, is given in Figure 3.9 (page 106).

4.6. IEF & HYDROLYTIC CHARACTERISTICS OF THE RECOMBINANT β-LACTAMASES

Crude extracts of each of the *E. coli* TG1 harbouring the recombinant plasmids were prepared. IEF of the β-lactamases produced by each strain was performed to ascertain whether the enzymes were of the correct pI compared to clinical isolations of the enzymes. Hydrolytic characteristics of the recombinant enzymes were determined against nitrocefin, ceftazidime and cefotaxime and compared to the characteristics of known β-lactamases. The summary of these verification procedures is given in the Table 3.8 (page 107).

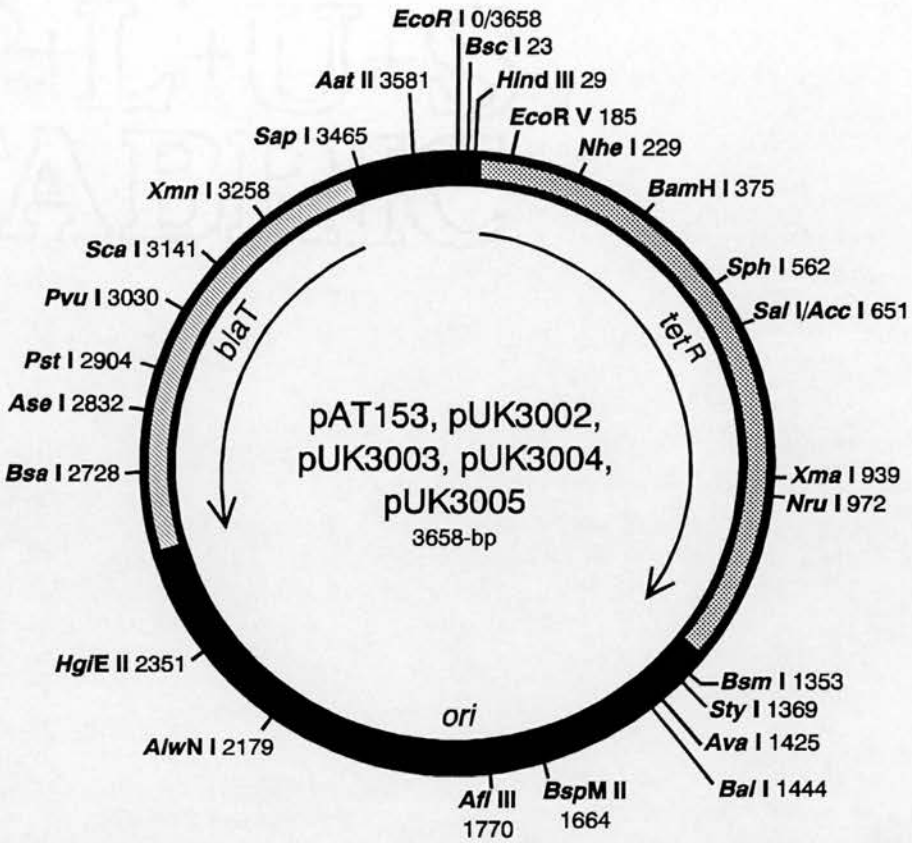

Figure 3.9: Restriction map of pAT153, pUK3002, pUK3003, pUK3004, & pUK3005

The map of the plasmids shows the position of the unique restriction sites for the endonucleases indicated according to various sources (Restriction endonuclease suppliers). Position 0 has been arbitrarily defined as the centre of the *Eco*R I site. Restriction site positions are given relative to the first base of the recognition sequence (except for *Eco*R I). The plasmids vary by one or two nucleotides. Abbreviations: *bla*T, TEM β-lactamase gene; *ori*, origin of replication; tet^R, tetracycline resistance gene. The following β-lactamase genes are carried by respective plasmids: *bla*T-1 (pAT153), *bla*T-12 (pUK3002), *bla*T-2 (pUK3003), *bla*T-17 (pUK3004), *bla*T-7 (pUK3005).

Enzyme	Plasmid	Isoelectric point (pI)		Relative Rates of Hydrolysis (%)‡			
		Expected	Actual	NCFN	PEN G	CAZ	CTX
TEM-12	pUK3002	5.25	5.25	100	174	47	79
TEM-2	pUK3003	5.6	5.6	100	1512	UM	UM
TEM-17	pUK3004	5.9	~5.5	100	1410	9.8	11
TEM-7	pUK3005	5.41	5.41	100	675	19	7.0
TEM-1	R1	5.4	5.4	100	56	UM	UM
TEM-2	R46	5.6	5.6	100	512	UM	UM
TEM-12	-	5.25	5.25	100	225	15	34
TEM-7	pIF100	5.41	5.41	100	809	20	12

Table 3.8: Isoelectric points & hydrolytic characteristics of recombinant β-lactamases

‡ Relative rate of hydrolysis relative to nitrocefin (100%). Abbreviations: CAZ, ceftazidime; CTX, cefotaxime; NCFN, nitrocefin; PEN G, penicillin G; UM, unmeasurable.

4.7. CONCLUDING REMARKS

Recombinant plasmids pUK3002, pUK3003, and pUK3005 were found to encode analogues of TEM-12, TEM-2, and TEM-7 respectively. It is apparent, however, from the results summarised in Table 3.8, that the recombinant plasmid pUK3004 did not encode an analogue of TEM-17 (expected pI 5.9). Unfortunately, time did not permit the *bla*T gene of pUK3004 to be re-sequenced to elucidate the nucleotide mutations with respect to *bla*T-1.

5. SEQUENCING OF THE TEM-E2$_{pUK721}$ β-LACTAMASE

Payne identified the mutant β-lactamase TEM-E2 from a clinical isolate [141, 151]. In 1982 the Neonatal ITU of Liverpool General Hospital had a number of deaths of neonates as a result of infections with a gentamicin resistant (but ceftazidime sensitive and producing TEM-1) *Kleb. oxytoca* 5446. Infections of *Kleb. oxytoca* 5446 were subsequently eradicated until the appearance of the gentamicin and ceftazidime resistant *Kleb. oxytoca* 5445 some months later. The particular strain characterised by Payne was isolated from the blood and cerebrospinal fluid of a baby in the unit. Ceftazidime resistance of *Kleb. oxytoca* 5445 was not thought to be enzymatically mediated as TEM-1 was the only β-lactamase identified at the initial isolation. Payne later revealed that the strain produced two β-lactamases, TEM-1 (pI 5.4) and an additional enzyme named, TEM-E2, with a pI of 5.3. Both β-lactamases had the same molecular weight (23.5×10^3) and were encoded by the same 141-kb plasmid, pUK721. It was suggested that the ceftazidime sensitive *Kleb. oxytoca* 5446 had remained in areas where it had been exposed to sub-MIC levels of ceftazidime and had led to the development of the ceftazidime resistant *Kleb. oxytoca* 5445. Payne had shown that TEM-1 could readily mutate *in vitro* to a TEM-E2-like enzyme under the selective pressure of ceftazidime [141, 161]. It has, therefore, been concluded that the selection of the TEM-E2 β-lactamase was a result of exposure of the TEM-1 producing strain to sub-MIC levels of ceftazidime. TEM-E2 is especially interesting because it shows significant similarity to other clinically isolated TEM-*type* enzymes (TEM-12, and CAZ-3), but predates these enzymes by at least five years. It is, therefore, the first example of a clinically isolated ESBL. It was decided that the nucleotide sequence of the TEM-E2 β-lactamase needed to be determined to ascertain its relationship to the later ESBL. If the TEM-E2 β-lactamase was analogous to TEM-12 it would have significant bearing on the perception of the relationships and development of the ESBL as described above (Section 3.2 page 94, and Figure 3.6 page 98).

5.1. DNA SEQUENCING STRATEGY

The strategy for the cloning and sequencing of TEM β-lactamase genes was based upon the a method designed by Dr Graham Disney and Mr Stephen Dove at Glaxo Group Research Ltd [174]. The cloning vector, pSG-2, was constructed by Disney

& Dove by modifying the cloning vector pBGS19+ (Kan^R *lacZ'*) to contain the TEM-1 promoter from pBR322 (Tn*3*) and the *rrn*BT_1T_2 terminator sequence from pKK233-2. PCR primer oligonucleotides were designed to amplify the ORFs of the TEM β-lactamase genes. These oligonucleotides encode restriction endonuclease recognition sequences (*Xba* I and *Sph* I) at their 5' ends to enable directional cloning of the PCR-amplified DNA into pSG-2. Counter-selection with ampicillin and kanamycin would select bacterial colonies containing a functional *bla*T ORF inserted between the promoter and terminator of pSG-2. Recombinant ampicillin resistance plasmids could then be sequenced for nucleotide changes within the ORF.

It was proposed that PCR-amplified DNA fragments from not only pUK721, but also pUK720 (TEM-E1), pUK722 (TEM-E3), pUK723 (TEM-E3) and pUK724 (TEM-E4), would be generated and cloned into pSG-2. The genes encoding TEM-E1, TEM-E3, and TEM-E4 had also remained uncharacterised at a level of the DNA sequence. Once PCR amplified DNA fragments were cloned, ampicillin resistant colonies would be used for the preparation of double-stranded plasmid DNA for sequencing. Each sequence would be determined from at least two separate PCR-amplification and cloning experiments to eradicate the possibility of the introduction of PCR-generated mutations being mistaken for the clinically generated nucleotide mutations.

5.2. PCR-AMPLIFICATION OF DNA FROM pUK721

Genomic DNA was isolated from overnight cultures of *E. coli* J53-2 (pUK721), or *E. coli* J62-2 (pUK720, pUK722, pUK723 or pUK724). Genomic DNA was then used in the PCR reactions to amplify 900-bp fragments from the *bla*T ORFs of the respective plasmids. Plasmid DNA from pSG-TEM-1 and pKK233-2 were used regularly for control reactions. It was found that generation of PCR fragments of TEM genes was temperamental by this method. It was suspected that mis-priming of PCR-primer oligonucleotides was responsible for the variation in success of PCR reactions.

A method for the generation of PCR-amplified DNA fragments direct from overnight cell cultures was developed. It was found that 1ml of overnight culture

of *E. coli* when washed once and resuspended in 1ml of 50mM Tris·Cl (pH 7·0) provided greater consistency in generation of 900-bp PCR-amplified TEM ORF fragments. One µl of the washed cell suspension was found to be the optimum volume for addition to PCR reactions. Volumes less than this did not yield optimum quantities of DNA. Volumes greater than 1µl gave temperamental results; it was thought this was because of the interference of bacterial proteins with the PCR reactions. PCR fragments were also generated from washed cell suspensions prepared from overnight cultures of bacteria that had been stored at 4°C for two days and from bacterial colonies removed from solid media after storage at 4°C for several days. The PCR reactions employing 'old' cultures, although possible, were found not to provide optimum quantities of PCR-amplified DNA.

5.3. Cloning of PCR-amplified DNA

The products of each PCR reaction were separated by agarose gel electrophoresis in 1×TAE buffer. The 900-bp fragments generated by successful reactions were excised and purified with the Geneclean II Kit (resuspended in a final volume of 10µl with TE buffer). Recovery of DNA from the agarose gel slices was verified by electrophoresis of 1µl of each purification in agarose gels in 1×TAE buffer. Five µl of each purified PCR fragment was restricted with *Xba* I and *Sph* I and ethanol precipitated to purify the restricted DNA from the components of restriction enzyme buffers and the restriction endonucleases.

The vector pSG-2 was prepared for insertion of the PCR-generated TEM ORFs. The recipient pSG-2 was restricted with *Xba* I and *Sph* I and the digested DNA fragments separated by agarose gel electrophoresis (in 1×TAE). The large (~4.4-kb) DNA fragment was excised from the agarose gel and purified by glass-milk purification.

The purified pSG-2 recipient fragment was then annealed and ligated overnight with the restricted 900-bp PCR fragment. The ligated DNA was used to transform competent Max Efficiency *E. coli* DH5α (Gibco BRL). Transformed bacteria were counter-selected with ampicillin, ampicillin and kanamycin, or ceftazidime. Colonies were screened for ampicillin, kanamycin and ceftazidime resistance depending on the original selecting antibiotics. Colonies with the required

phenotype (Kan^R Amp^R) were used to prepare plasmid DNA for restriction analysis with *Eco*R I, *Hin*d III, *Sph* I and *Xba* I. Plasmid DNA exhibiting the correct restriction characteristics was used to prepare double-stranded plasmid DNA for DNA sequencing.

Apart from the initial problems with PCR amplification of TEM ORFs, that were improved by substituting washed cell suspensions for total genomic DNA preparations, there were problems with the cloning of the PCR-fragments. Contaminating organisms were found to be a persistent problem on the solid media used for counter-selecting transformants. The multi-resistant organisms were partially irradicated when the source of the contamination was attributed to surface-drying of plates in a 37°C incubator.

Ligation and transformation efficiency throughout each of the cloning experiments was unexpectedly low (maximum of one to three colonies per plate for the insertions that worked). Disney & Dove had similar problems with the cloning of the *bla*T-1, *bla*T-3, *bla*T-5, *bla*T-7, and *bla*T-9 ORFs. The manufacturers of the competent *E. coli* DH5α recommend dilution of ligation reactions prior to transformation, as ligation reaction components can interfere with the transformation process. It was found that dilution of control ligation reactions did not greatly affect the efficiency of transformation. Ligation reaction components were, therefore, not considered responsible for the low efficiency. One of the restriction enzymes, *Sph* I was therefore suspected of low cutting efficiency of PCR-amplified DNA. The *Sph* I recognition sequence (···GCATGC···) is located very close to one end of the PCR fragment (three nucleotides from the 5'-end of the PCR primer oligonucleotide); because of this location, digestion of PCR-amplified DNA with this enzyme was suspected not to be as efficient as the digestion of intact plasmid DNA. Complete digestion of pSG-2 DNA was regularly observed with *Xba* I and *Sph* I.

The TA Cloning System (Invitrogen Corporation) was used to try and circumvent the restriction and ligation problems encountered. The kit was designed for the one-step cloning of PCR-amplified DNA into a specially prepared plasmid vector (pCR II). Thermostable polymerases (such as the *Taq* polymerase used in the PCR reactions) have a non-template dependent activity that adds single deoxyadenines

to the 3'-end of all duplex DNA. The poly-deoxyadenine 'overhangs' were used to try and clone the PCR fragment into the pCR II vector (supplied as a linear molecule with 3'-T overhangs). An aliquot of the PCR reaction mixture was diluted according to the TA Cloning System manufacturer's protocol and annealed and ligated overnight with the linear pCR II DNA. The product of the ligation was then used to transform the competent *E. coli* INVαF' supplied with the kit. Colonies were counter-selected on kanamycin (the pCR II vector confers Kan^R & Amp^R). Presence of a PCR fragment insert within the pCR II vector was also ascertained with concurrent Lac (blue-white) selection of colonies. The pCR II self-ligation control yielded some white colonies together with the expected blue colonies. White colonies from the cloning experiment were selected for isolation and restriction analysis. Restriction analysis with *Eco*R I (the MCS of pCR II is palindromic, therefore, restriction with a single enzyme will excise any inserted DNA fragment) revealed that none of the white colonies harboured a pCR II-PCR fragment construct as expected from the results of the self-ligation control experiment. The use of this system was reputed to have been temperamental at the time of the experiment.

PCR-amplified pUK721 ORF DNA was also cloned into the pBGS18+ vector. PCR DNA amplified from pUK721 and pBGS18+ DNA were restricted separately with *Xba* I and *Sph* I. Restricted DNA fragments were separated by agarose gel electrophoresis and the 900-bp PCR fragment band and 4.4-kb pBGS18+ band were excised and purified with the Geneclean II Kit (Bio 101). The purified digested PCR DNA and pBGS18+ DNA were annealed and ligated overnight. Aliquots of the ligation mixture were used to transform Max Efficiency *E. coli* DH5α. Colonies were counter-selected with kanamycin, and insertion of the PCR DNA into the MCS of pBGS18+ was identified by Lac selection. White colonies were selected for isolation and restriction analysis. Restriction with *Eco*R I and *Hin*d III revealed the presence of a 900-bp insert within pBGS18+ of one of the selected colonies. Plasmid DNA, prepared from these colonies, was used to re-transform Max Efficiency *E. coli* DH5α (Gibco BRL). All transformants underwent Lac selection with concurrent counter-selection with kanamycin. Transformed colonies were all white confirming the purity of the pBGS18+-PCR fragment construct plasmid preparation.

5.4. DNA SEQUENCING OF CLONED PCR-AMPLIFIED DNA

Sequencing grade double-stranded plasmid DNA was prepared, with the Qiagen Midi-Prep Kit, from each of the colonies that were verified to contain a construct with a 900-bp DNA insert. The plasmid DNA was denatured in alkaline solution and the relevant oligonucleotide sequencing primer (see Table 3.9) annealed. DNA sequencing of the PCR-amplified DNA insert was carried out according to the method described in Chapter 2 for sequencing of double-stranded DNA templates.

A summary of all of the PCR-experiments and the sequencing of PCR inserts, including the pCR II and pBGS18+ cloning experiments, is given in Table 3.10 (page 114). All the DNA sequenced was shown to be derived from the gene of the TEM-1 β-lactamase. No PCR-amplified TEM-ORF was successfully cloned from fragments generated from pUK720 or pUK724. It was expected, as pUK721 encoded two TEM-β-lactamase genes, that PCR would generate equal copies of each ORF and, therefore, there was equal probability of cloning the *bla*T-1 and *bla*T-E2 ORF. Cloning and selection of constructs on ampicillin or ceftazidime would have selected for plasmids containing the TEM-1 and TEM-E2 ORFs, and the TEM-E2 ORF respectively. No ceftazidime resistant *E. coli* DH5α colonies were isolated by this procedure. Of the three plasmid-constructs sequenced (two Amp^R pSG-2 constructs and a *lac*$^-$ pBGS18+ construct) no nucleotide differences from *bla*T-1 were observed.

Primer‡	Sequence§	Position†	Size
Bla-(S)391	5'-A GAA CGT TTT CCA ATG-3'	391-406	16-mer
Bla-(S)606	5'-CC AAC TTA CTT CTG AC-3'	606-621	16-mer
Bla Seq 6	5'-TAA CCC TGA TAA ATG C-3'	167-182	16-mer
Bla Seq 7	5'-CAA GAG CAA CTC GGT C-3'	464-479	16-mer
Bla Seq 8	5'-ACA ACG TTG CGC AAA C-3'	764-779	16-mer

Table 3.9: Oligonucleotide primers for DNA sequencing

‡ Primer nomenclature refers to location of first (5') nucleotide. § Oligonucleotides are sense strand analogues. † 5'→3' binding range of sense oligonucleotides to non-sense strand of *bla*T-1 (DNA sequence numbering according to Sutcliffe (1978) [150])

		β-Lactamase					Total
		TEM-E1	TEM-E2	TEM-E3	TEM-E3	TEM-E4	
Plasmid:	Name	pUK720	pUK721	pUK722	pUK723	pUK724	-
	Size	31-kb	141-kb	110-kb	100-kb	56-kb	-
PCR‡	DNA	8	17	13	13	11	62
	Cells	3	36	10	9	9	67
Successes§:	DNA (%)	3 (38)	4 (24)	5 (38)	5 (38)	6 (55)	23 (37)
	Cells (%)	1 (33)	23 (64)	2 (20)	2 (22)	2 (22)	30 (45)
Cloning†:	Purification	3	20	3	4	4	33
	Ligations	7	22	5	8	7	49
	Colonies	2	6	4	3	3	17
Sequencing*		-	3	2	2	-	7

Table 3.10: Summary of PCR-amplification experiments of *bla*T ORFs from pUK720, pUK721, pUK722, pUK723, pUK724

‡ Number of PCR amplifications: DNA, from total genomic DNA preparations; Cells, from whole washed cell suspensions. § Number of PCR experiments that generated ~900-bp DNA fragments (percentage of PCR experiments that generated a fragment in brackets). † Number of cloning experiments: Purification, number of PCR-amplified DNA fragments purified in preparation for cloning; Ligations, number of overnight ligation experiments to insert PCR-amplified DNA into a recipient vector; Colonies, number of overnight ligation experiments that produced colonies (of the correct phenotype) after transformation of *E. coli* DH5α. * Number of recombinant plasmids, with the correct construction, that had the *bla*T ORF DNA sequence fully elucidated.

5.5. SINGLE-STRANDED CONFORMATIONAL POLYMORPHISM

Single-stranded conformational polymorphism (SSCP), when combined with PCR-amplification of the target DNA sequence (called PCR-SSCP) has been used to detect single-nucleotide changes in several hundred bases [238]. In SSCP analysis a mutated sequence is detected as a change of mobility in polyacrylamide gel electrophoresis, as a result of an altered folded structure. Under non-denaturing conditions, single-stranded DNA has a conformation stabilised by intra-strand interactions. As a consequence, the conformation, and therefore the mobility, is dependent on the DNA sequence [239]. After amplification of the DNA (from washed cell suspensions) by PCR, including ^{32}P-α-dCTP to radio-label the product, the amplimers were heat denatured and conformers separated by polyacrylamide gel electrophoresis with the Mutation Detection Enhancement (MDE) Gel (AT Biochem Inc.). Electrophoresis was carried out at constant power (8 Watts) at room temperature for 14 hours, the recommended conditions to separate PCR amplimers of up to 1-kb which differ by a single nucleotide. After electrophoresis the gel was dried under vacuum for 60 minutes at 70°C then autoradiographed for 12 hours with intensifying screen at –70°C.

The developed autoradiographs showed that PCR-amplified DNA derived from *bla*T-$10_{pJPQ100}$ and *bla*T-$12_{pUK3002}$ formed only two SSCP bands whereas pUK721-derived PCR-DNA exhibited three bands (Figure 3.10 page 116). Other PCR-amplimers derived from plasmids encoding a single β-lactamase also demonstrated only two bands (not shown). The two bands produced from *bla*T-$12_{pUK3002}$ correspond to the upper two bands presented by the PCR-DNA from pUK721.

The PCR-SSCP analysis has demonstrated that a small difference between the two β-lactamase genes present on pUK721 exists. From the comparison with the bands derived from the recombinant pUK3002, it would appear that one of the conformers from pUK721 is closely related to TEM-12. The significance of the number of bands produced, or migration distance of each conformer generated, by PCR-SSCP has not been fully elucidated by the various pioneers of the technique. The analysis is only useful to show that differences between closely-related DNA species exist.

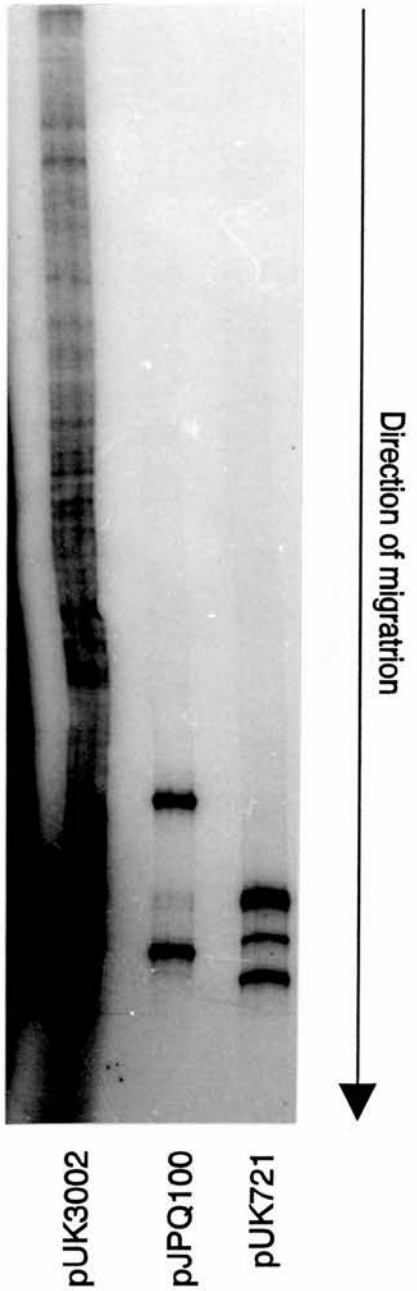

Figure 3.10: PCR-SSCP analysis of pUK721, pUK3002 & pJPQ100

The plasmids used for generation of ^{32}P-labelled PCR DNA fragments for analysis encode the following TEM β-lactamase genes: *bla*T-10 (pJPQ100), *bla*T-12 (pUK3002), *bla*T-1 & *bla*T-E2 (pUK721).

5.6. DIRECT DNA SEQUENCING OF PCR-AMPLIFIED DNA

The results of the PCR-SSCP analysis suggest that PCR-amplification of DNA from pUK721 generates ~900-bp fragments from both the *bla*T-1 and *bla*T-E2 gene. It is, therefore, an anomaly that no ceftazidime resistant clones were produced from the insertion of PCR-fragments into pSG-2. In theory, if both pUK721 TEM β-lactamase genes are amplified by the PCR, direct sequencing of the PCR products should demonstrate different nucleotide bands at the same position on a DNA sequencing gel; each band corresponding to the respective sequence of each PCR-generated species present in the sequencing reaction. Various strategies were tried to obtain the DNA sequence of TEM β-lactamase genes direct from PCR-amplified DNA fragments. The major problem of sequencing short segments of double-stranded DNA, like PCR-amplimers, is the tendency of the templates to reanneal. Each method employed (excluding the initial procedure of double-stranded DNA sequencing) incorporates specific steps to help minimise the reannealing of PCR-DNA strands. All sequencing reactions included ^{35}S-α-dATP or ^{33}P-α-dATP to radiolabel the reaction products.

DOUBLE-STRANDED DNA SEQUENCING OF PCR-PRODUCTS

Sequencing of the purified 900-bp PCR-amplified *bla*T fragments was attempted following a standard method for the sequencing of double-stranded plasmid DNA (as described in Chapter 2 [221, 225]). After polyacrylamide gel electrophoresis of the sequencing reaction products the dried gels were autoradiographed overnight (~12 hours). Although control reactions employing the plasmids pKK233-2 or pBR322 generated readable DNA sequences, no PCR-amplimers gave any sequences after polyacrylamide gel electrophoresis of the reaction products.

MCPHERSON METHOD FOR THE DIRECT SEQUENCING OF PCR-PRODUCTS

The method for direct sequencing of PCR-products, as described by McPherson *et al.* [226], was followed with purified PCR-generated DNA from various *bla*T genes. The procedure requires the heat denaturation of the double-stranded PCR-amplimers in the presence of sequencing primer, followed by snap-freezing of the solution on a dry-ice. After thawing the solution at room temperature, the sequencing reactions were carried out as described in Chapter 2 following the

method recommended by the manufacturers of Sequenase (USB). After separation of the sequencing reaction products by polyacrylamide gel electrophoresis the gels were dried and autoradiographed for ~12 hours, but no readable DNA sequences were observed. DNA sequences that were produced suffered from secondary structure artefacts (see Figure 3.11 page 119). Control sequencing reactions using double-stranded plasmid DNA (pKK233-2 or pBR322) gave readable DNA sequences on each occasion.

DIRECT SEQUENCING OF PCR-PRODUCTS USING DIMETHYL SULPHOXIDE

The use of dimethyl sulphoxide (DMSO) can help overcome the problem of reannealing of PCR-DNA strands [227]. The procedure described by Winship [227] was followed. The purified PCR-amplified DNA from each *bla*T gene was mixed with 20pmoles of the required sequencing primer and denaturation buffer to achieve final concentrations of 10%V/v DMSO, 50mM NaCl, 40mM Tris·Cl (pH 7.5) and 25mM $MgCl_2$. The double-stranded DNA was heat denatured and then snap-frozen on dry-ice. The solution was thawed and sequencing reactions carried out according to the procedures recommended for use with Sequenase Version 2.0 [225] except for the addition of DMSO to 10%V/v to the extension and termination reactions. The sequencing reaction products were separated by electrophoresis, dried, and autoradiographed for ~12 hours. All control sequencing reactions using double-stranded plasmid DNA (pBR322 or pKK233-2) gave readable DNA sequences. Readable DNA sequences were obtained from the PCR-amplified DNA samples but with considerable interference from secondary structure artefacts (see Figure 3.11 page 119). Slight alterations in the reaction procedures improved sequence readability and reduced the number of artefacts by a small degree.

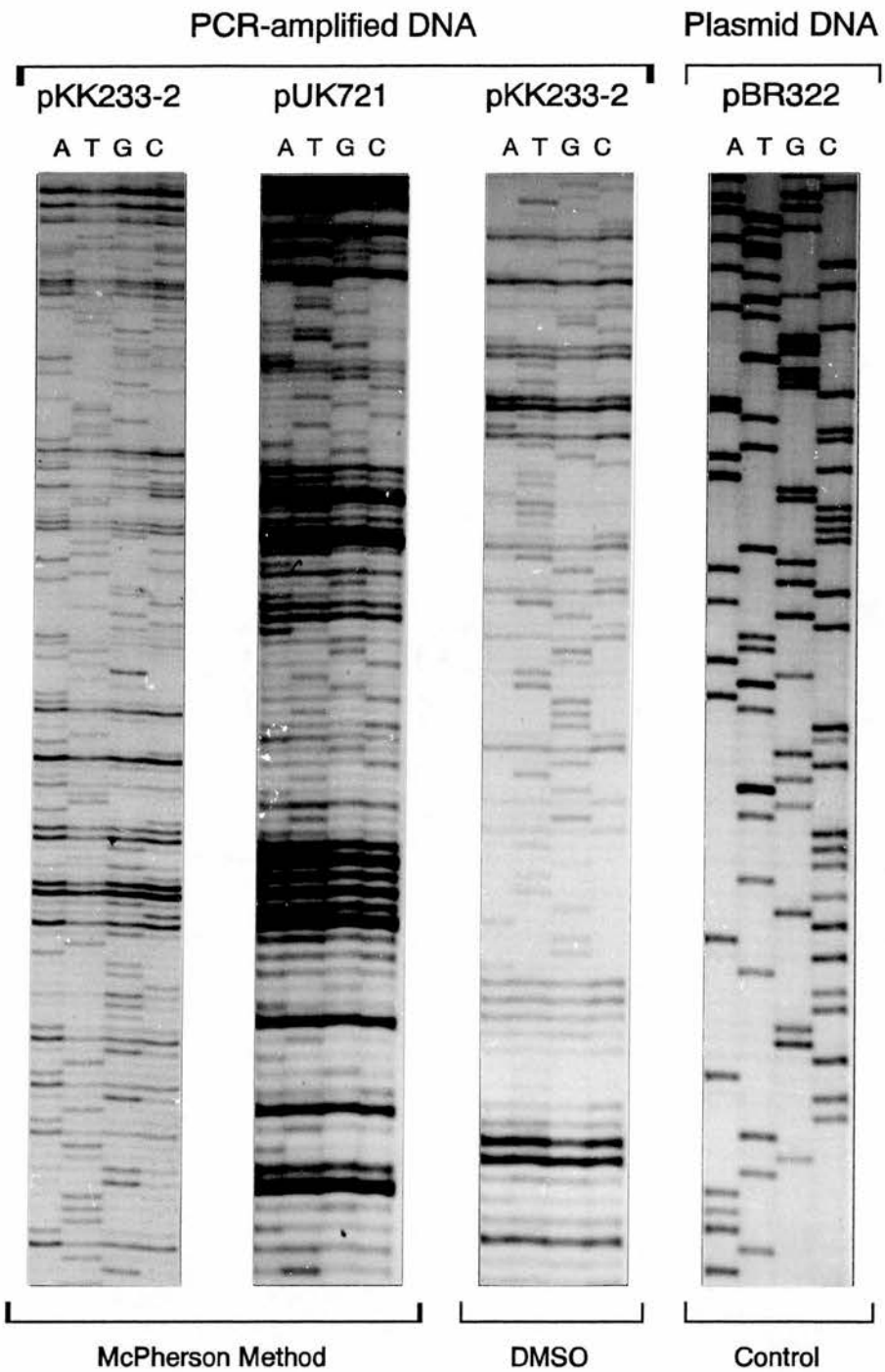

Figure 3.11: Autoradiograph results from direct-sequencing of PCR-products

Results from the direct sequencing of PCR-amplified DNA from pKK233-2 and pUK721 using the McPherson Method [226] and DMSO method [227]. The control reactions employed sequencing of double-stranded plasmid DNA with reactions conducted as described in Chapter 2.

DIRECT SEQUENCING OF PCR-PRODUCTS USING SINGLE-STRANDED DNA BINDING PROTEIN

Single-stranded DNA binding protein (SSB) has a high affinity for single-stranded DNA but does not bind to double-stranded DNA [240]. The presence of SSB in sequencing reactions helps prevent secondary structure formation and reassociation of PCR-DNA strands. Use of SSB, less dependent on ionic strength for binding than other single-stranded DNA binding proteins, was tried by following the method of Wanner, Tilmans & Mischke [228]. PCR-DNA was heat denatured in the presence of sequencing primer then snap-frozen on dry-ice. The solution was thawed and SSB (0.5μg) was added prior to the primer extension and radiolabelling step. The SSB was inactivated prior to running the sequencing gel by adding 0.1μg of proteinase K and incubating at 65°C for 20 minutes. Reaction products were then separated by polyacrylamide gel electrophoresis, followed by autoradiography of the dried gel for ~12 hours. No DNA sequence was observed on autoradiograph films, whereas control sequences determined from double stranded plasmid DNA (pAT153) all worked. SSB from two different manufacturers (Pharmacia and USB) were used in a number of sequencing experiments but with no success. The initial denaturation and snap-freezing step was found to be the most crucial part of the procedure, but readable sequences were still unattainable.

DIRECT SEQUENCING OF PCR-PRODUCTS WITH DYNABEADS

A biotin ligand was incorporated into the 5′-terminus PCR-primer (Biotin-Bla 4' had the same DNA sequence as the Bla 4' PCR-primer described in Chapter 2). The strategy for direct DNA sequencing from PCR-amplified DNA using Dynabeads is outlined in Figure 3.12 (page 121). Following amplification, the double-stranded PCR-product was immobilised on Dynabeads M-280 Streptavidin (Dynal, Norway). Dynabeads M-280 Streptavidin consist of uniform, superparamagnetic, polystyrene beads, 2.8μm in diameter. Streptavidin is covalently attached to the bead surface. Once the PCR-product was selectively captured and immobilised with the Dynabeads, the DNA was isolated by magnetic separation. Elution of the non-biotinylated amplimer, with alkali, provided a single-stranded DNA template that was sequenced using the Sequenase T7 DNA polymerase.

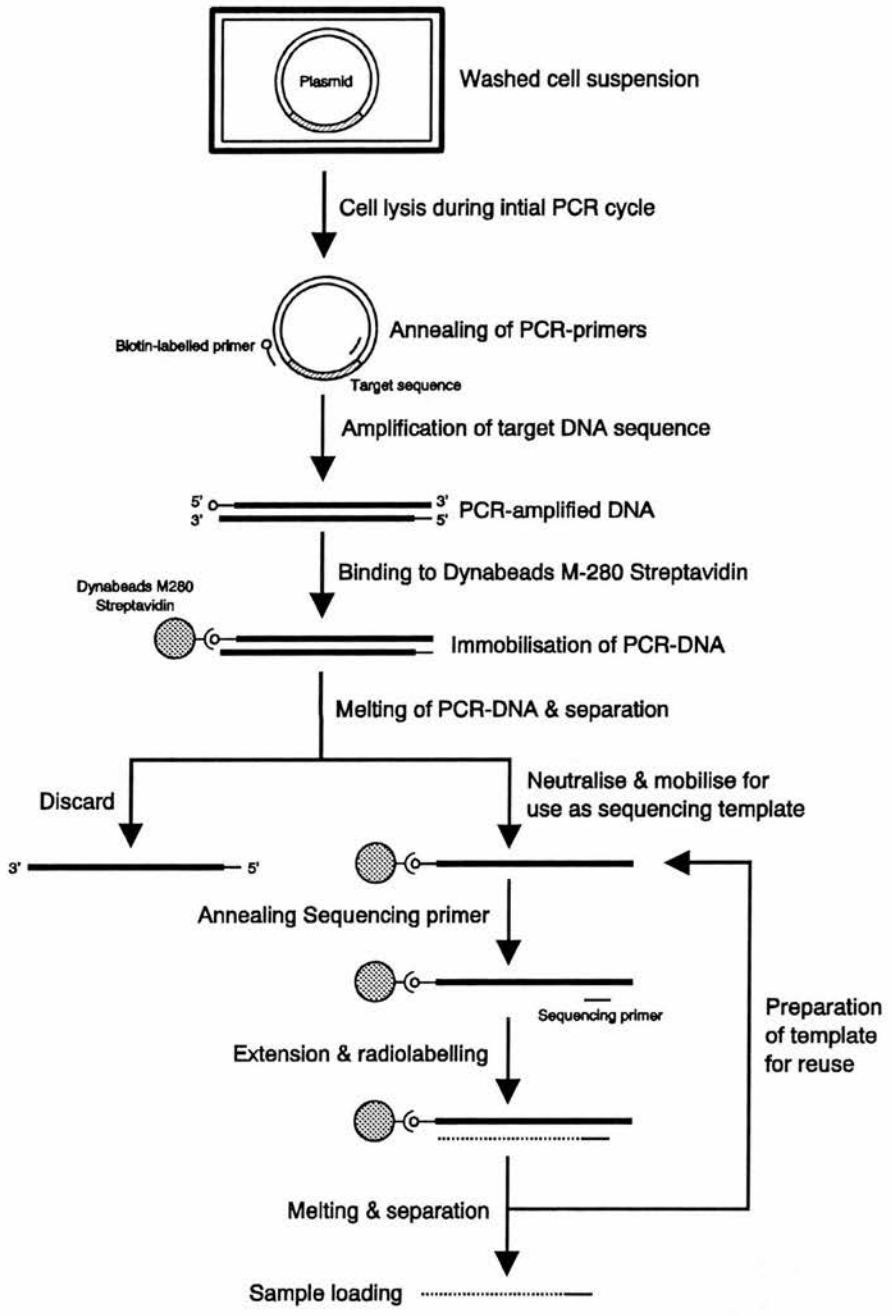

Figure 3.12: Strategy for the direct sequencing of PCR-products using Dynabeads

Simplified scheme for the PCR-amplification of TEM ORFs using the biotin labelled 'Biotin-Bla 4'' PCR-primer and subsequent immobilisation with Dynabeads M-280 Streptavidin (Dynal, Norway). Once immobilised denaturation allows removal of one DNA strand. The Dynabead-labelled strand can then be used as a single-stranded template for DNA sequencing procedures. After sequencing reactions the template can be washed and re-used in further reactions.

The template DNA from a single PCR reaction can be re-used: prior to loading onto the polyacrylamide gel the template was salvaged from the sequencing reaction mixture by magnetic separation. Re-use of the template required stringent denaturation and washing steps to remove all of the radiolabelled sequencing reaction products. Sequencing reaction products were separated by polyacrylamide gel electrophoresis, and the dried gels autoradiographed for ~12 hours. Sequencing reactions were shown to be very successful by this method. 'Secondary bands' were observed with recycled templates if the DNA had not been washed sufficiently after salvage.

From the DNA sequences generated from pUK721 by this method (Figure 3.13 page 123), two nucleotide bands at DNA residue number 692, corresponding to adenine and cytosine were observed. This shows that both the codon for Arg-164 (CGT) and Ser-164 (AGT) are present in the PCR-DNA. Relative intensities of the two simultaneous bands were found to vary from approximately equal to very faint A^{692} bands. No other anomalous adenine bands were observed elsewhere in the DNA sequence. By separating the adenine and cytosine lanes by at least one other nucleotide lane the possibility of simultaneous bands resulting from leakage of reaction mixes between lanes was eliminated. Control reactions using PCR-DNA generated from pAT153 exhibited no such duplicate bands over the same nucleotide sequence region.

This experiment has shown that both nucleotides, which separate *bla*T-1 and *bla*T-E2, are present on pUK721. My results suggest that TEM-E2 is the same as TEM-12, both of which would appear to vary from TEM-1 by a single amino acid substitution encoded by the single nucleotide mutation from cytosine-692 to adenine-692.

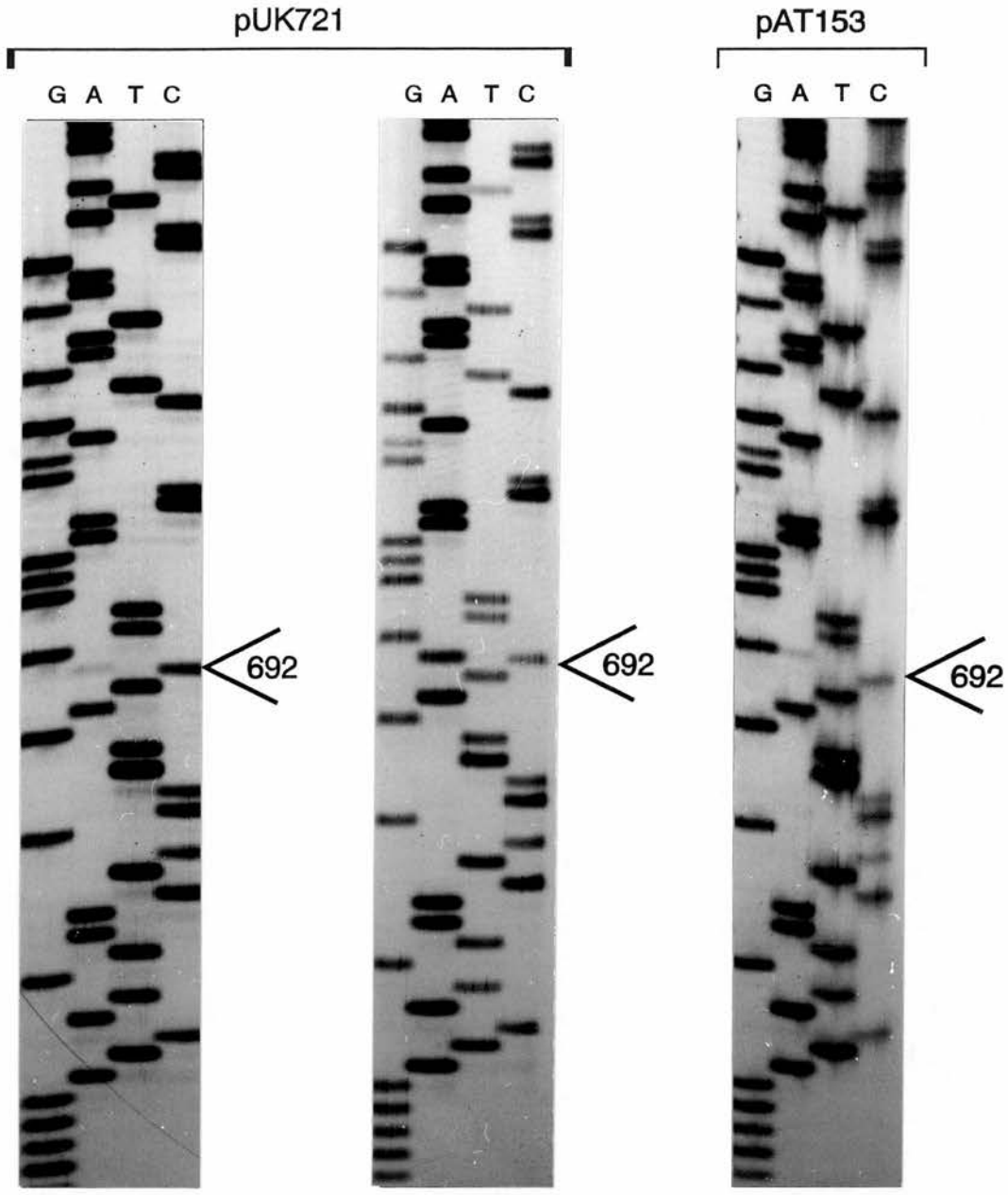

Figure 3.13: Results of the direct sequencing of PCR-products from pUK721 using Dynabeads

The photographs of the autoradiographs are marked to show nucleotide position 692. From the control sequencing reactions generated from pAT153 (right) only a single nucleotide (cytosine) was observed at position 692. PCR-amplified DNA from pUK721 demonstrated simultaneous bands (adenine & cytosine) at nucleotide position 692 which varied in band intensity from weak adenine (left) to approximately equal (middle).

6. THE EFFECT OF PLASMID COPY NUMBER ON THE MIC OF β-LACTAM ANTIMICROBIAL AGENTS

The MICs of the β-lactam agents ampicillin, amoxycillin, amoxycillin/clavulanic acid (ratio 2:1), ceftazidime and cefotaxime were determined against a number of TEM-1 producing *E. coli* strains at three different inoculum sizes. Plasmid copy number per chromosome varies from about one up to 100. Three inoculum sizes were used: 10^4 cfu/spot, 10^5 cfu/spot, and 10^6 cfu/spot. This experiment was carried out in order to gauge the effect of the copy number of the plasmids that encode the TEM β-lactamases upon the resistance of the host organism against the penicillins, 3GCs and the penicillin/β-lactamase inhibitor combinations. The results of the antibiotic susceptibilities of the organisms described in Table 3.11 are listed in Table 3.12 (page 125).

From Table 3.12 it can be seen that a decrease in inoculum size corresponds to a decrease in MIC of the test antibiotic. In general, the decrease of inoculum size from

Plasmids			Strain details	
Name	Size (kb)	Copy No.‡	Strain§	Source†
pUC8	2.7	50-100	JM108	SA1489
pUC18	2.7	50-100	JM83	SDB73
pAT153	3.7	30-60	TG1	SDB101
pBR322	4.4	~20	1009	SA473
R6K	39.9	~20	J53-2	SA471
R1	96.1	1-2	J62-2	SA17

Table 3.11: Details of plasmids & host organisms used to determine the effect of copy number on the MIC of β-lactam antibiotics

‡ Approximate copy number of plasmid per cell. § Strain of *E. coli* used as host organism for plasmid. † Source of host organism (culture collection number: SA prefix refers to S.G.B. Amyes' culture collection; SDB prefix refers to S.K. Du Bois' culture collection).

10^6 cfu/ml to 10^4 cfu/ml only decreases the MIC of the antibiotic by a single dilution. Susceptibilities of the host organisms to ampicillin and amoxycillin are approximately equal for each organism tested. The addition of clavulanic acid to amoxycillin (ratio 1:2 respectively) reduces the concentration of the penicillin required to inhibit growth of the test organisms. Increase in the number of copies of each plasmid per chromosome, hence a proportional rise in TEM-1 expressed, does not greatly affect the MIC of the antibiotics examined. Increase in resistance to the 3GCs (ceftazidime and cefotaxime) of organisms hosting high copy number plasmids (pUC8, pUC18) is not vastly different from that of low copy number plasmids (R6K, R1), in terms of clinical resistance.

Plasmid	Inoculum	MIC (mg/l)				
	(cfu/spot)	AMP	AMX	AMX/CLAV	CAZ	CTX
pUC8	10^6	>4096	>4096	32	0.5	0.125
	10^5	>4096	>4096	16	0.5	0.063
	10^4	>4096	>4096	16	0.25	0.063
pUC18	10^6	>4096	>512	32	0.5	
	10^5	3072	>512	32	0.25	N/D
	10^4	2048	512	16	0.063	
pAT153	10^6	>6144	>6144	32	0.5	0.125
	10^5	6144	5120	32	0.5	0.063
	10^4	5120	4096	16	0.125	0.031
pBR322	10^6	>6144	>6144	32	0.25	0.063
	10^5	5120	3072	16	0.25	0.031
	10^4	4096	3072	16	0.125	0.031
R6K	10^6	>4096	>4096	16	0.125	0.063
	10^5	2048	4096	16	0.125	0.031
	10^4	2048	4096	16	0.063	0.031
R1	10^6	3072	3072	8	0.063	0.063
	10^5	<512	512	8	<0.125	0.031
	10^4	<512	512	8	<0.125	0.031

Table 3.12: The effect of plasmid copy number and inoculum on β-lactam antibiotic susceptibility

Abbreviations: AMP, ampicillin; AMX, amoxycillin; AMX/CLAV, amoxycillin/clavulanic acid (ratio 2:1); CAZ, ceftazidime; CTX, cefotaxime; N/D, not measured.

7. THE EFFECT OF "REVERSE" SELECTIVE PRESSURE ON THE TEM-β-LACTAMASES

Sowek *et al.* (1991) [241] described the effects of lysine substitution at amino acid positions 104 and 240 and the substitution with serine at position 164 on the enzyme TEM-1_{pTZ18R}. Sowek and her colleagues found that if glutamic acid-104 or -240 were substituted with a lysine, in combination with a serine-164 substitution, the resultant enzyme had enhanced cephalosporin hydrolysis and activity. The paper describes a number of recombinant enzymes that are equivalent to clinically isolated β-lactamases, namely TEM-10, TEM-12, TEM-17 and TEM-26. Analysis of the enzymological data presented by Sowek *et al.* revealed some interesting features that appear to have been ignored by the paper's authors. A summary of the relevant data is given in Table 3.13 (page 127). The data have been arranged according to the proposed inter-relationship of the TEM β-lactamases (Section 3.2 page 94 and Figure 3.6 page 98) in Figure 3.14 (page 128) and Figure 3.15 (page 129). The Michaelis constant, K_m, values (Figure 3.14) do not appear to show any clear trend from TEM-1 to TEM-5 or TEM-9. It can be seen that K_m for benzylpenicillin decreases, hence affinity for the substrate increases. Michaelis constant values for the other two substrates show a general increase, describing a lower affinity. Turnover number, k_{cat}, is a more sensitive gauge of enzyme efficiency. If the k_{cat} values of each enzyme for the three substrates are compared, (Figure 3.15) in relation to the proposed evolution of the TEM β-lactamases, two trends are observed:

- Turnover numbers of the enzymes increase for the 3GCs with each additional mutation away from the wild-type enzyme (TEM-1).
- As turnover number increases to the 3GCs, k_{cat} decreases for benzylpenicillin.

This analysis of the data shows that selective pressure from a simple penicillin, for example penicillin G, ampicillin or amoxycillin, may select out the back-mutations.

		Enzyme				
		TEM-1	**TEM-10**	**TEM-12**	**TEM-17**	**TEM-26**
Plasmid		pTZ18R	pRDD006	pRDD004	pRDD002	pRDD005
Amino acid:	104	Glu	Glu	Glu	LYS	LYS
	164	Arg	SER	SER	Arg	SER
	240	Glu	LYS	Glu	Glu	Glu
MIC (mg/l)	PEN G	>128	>128	>128	>128	>128
	CAZ	0.25	32	2	0.25	64
	CTX	0.03	0.25	0.03	0.03	0.25
K_m (μM)	PEN G	26	5.7	16	19	5.8
	CAZ	80	190	260	150	150
	CTX	450	32	230	470	84
k_{cat} (sec^{-1})	PEN G	520	17	54	370	32
	CAZ	0.0016	17	3.4	0.067	57
	CTX	0.25	0.39	2.4	2.5	2
I_{50} (nM)‡	CLAV	90	9.5	18	30	6.2

Table 3.13: Characteristics of the recombinant TEM-β-lactamases described by Sowek *et al.* (1991) [241].

‡ Enzyme and inhibitor incubated for 5 minutes at 25°C before the reaction was initiated by the addition of cephaloridine to 1mM. Data from: Sowek *et al.* (1991) [241]. Abbreviations: Arg, arginine; CAZ, ceftazidime; CTX, cefotaxime; Glu, glutamic acid; Lys, lysine; PEN G, penicillin G; Ser, serine. Amino acids annotated entirely in capital letters indicate the substituted residues in the mutant enzymes. Numbering of amino acid residues according to the recommendations of Ambler *et al.* (1991) [158]

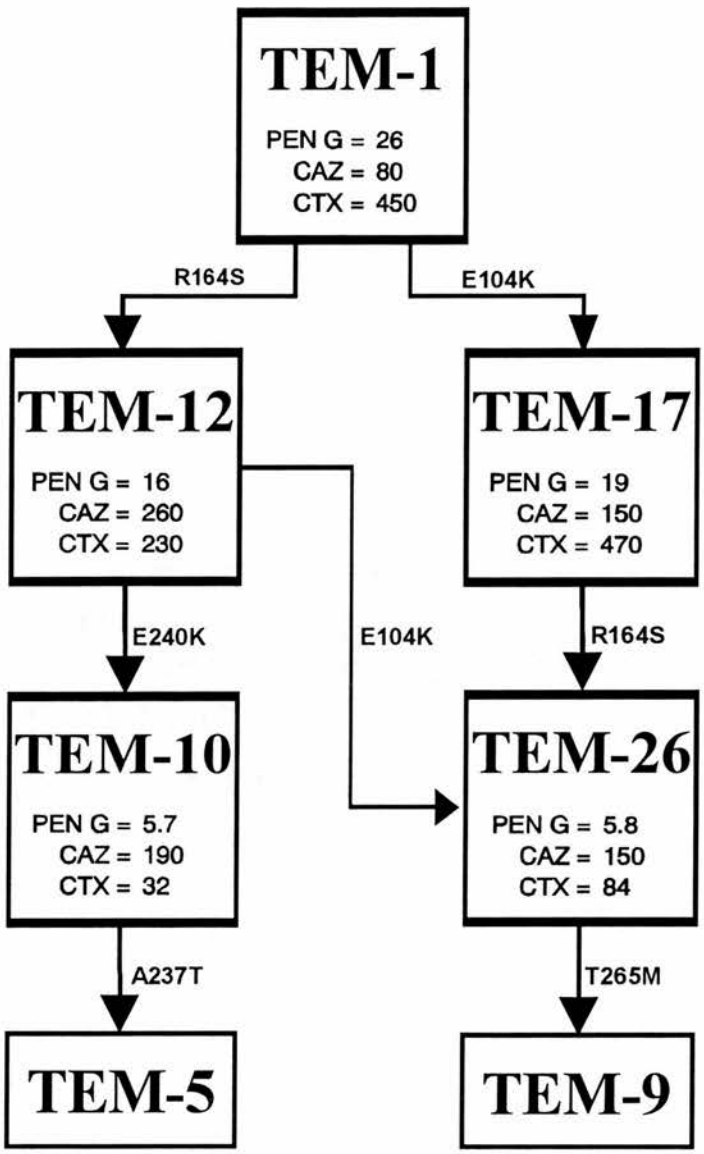

Figure 3.14: Comparison of the values of the Michaelis constant of various TEM β-lactamases for three β-lactam antibiotics

All values of Michaelis constant, K_m, given in μmoles dm^{-3} (μM). K_m values taken from Sowek *et al.* [241]. Each arrow represents a single amino acid change facilitated by a single nucleotide change in the β-lactamase gene. One letter amino acid notation according to the IUPAC-IUB Commission on Nomenclature [237]: A, alanine; E, glutamic acid; K, lysine; M, methionine; R, arginine; S, serine; T, threonine. Numbering of amino acid residues according to the recommendations of Ambler *et al.* (1991) [158]. Abbreviations: CAZ, ceftazidime; CTX, cefotaxime; PEN G, penicillin G.

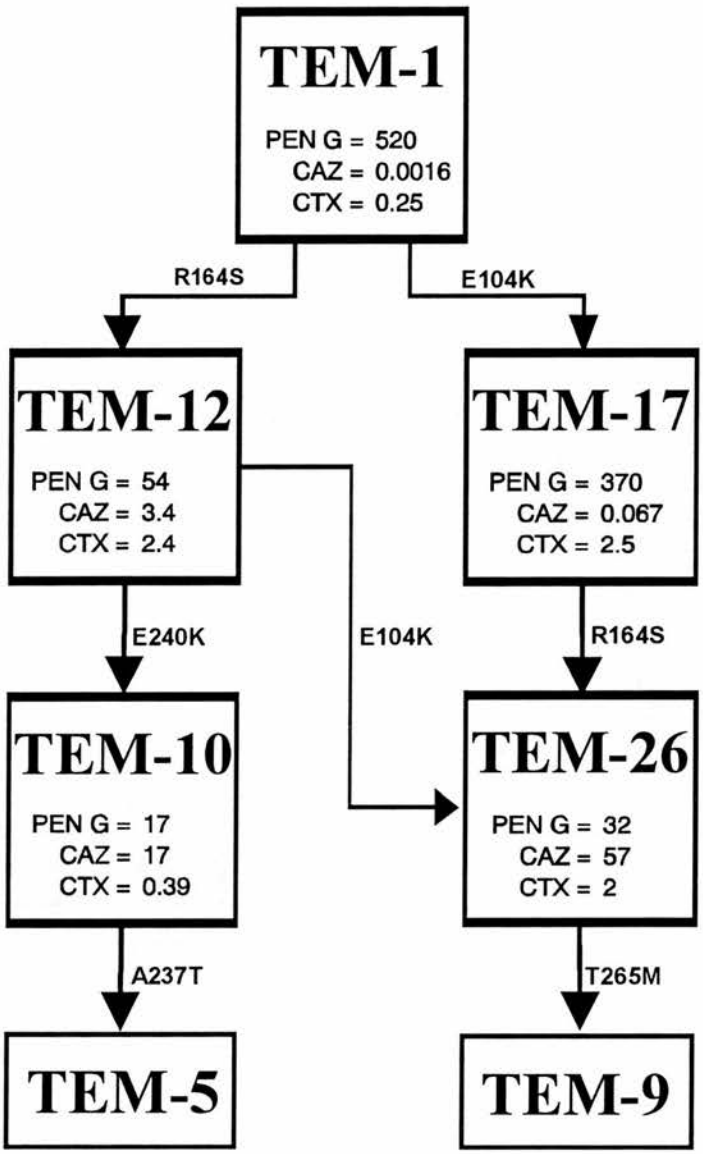

Figure 3.15: Comparison of the turnover number of various TEM β-lactamases for three β-lactam antibiotics

All values of turnover number, k_{cat}, given in seconds^{-1}. k_{cat} values taken from Sowek *et al.* [241]. Each arrow represents a single amino acid change facilitated by a single nucleotide change in the β–lactamase gene. One letter amino acid notation according to the IUPAC-IUB Commission on Nomenclature [237]: A, alanine; E, glutamic acid; K, lysine; M, methionine; R, arginine; S, serine; T, threonine. Numbering of amino acid residues according to the recommendations of Ambler *et al.* (1991) [158]. Abbreviations: CAZ, ceftazidime; CTX, cefotaxime; PEN G, penicillin G.

7.1. ENZYME KINETIC ANALYSIS OF THE TEM-1, TEM-5, TEM-9, TEM-10, TEM-12 & TEM-26 β-LACTAMASES

The enzyme kinetics of the TEM-1_{R1}, TEM-5_{pCFF14}, TEM-9_{pMG228}, TEM-$10_{pJPQ100}$, TEM-12 and TEM-$26_{pJPQ101}$ β-lactamases were determined in order to confirm the observations of Sowek *et al.* [241] and to analyse the properties of the terminus enzymes (TEM-5 and TEM-9).

The maximum velocity of hydrolysis, V_{max}, and the Michaelis constant, K_m, were determined for the substrates nitrocefin, penicillin G, ceftazidime and cefotaxime. A summary of the results of each kinetic analysis is given in Table 3.14 (page 131). The β-lactamase TEM-17 was not available for analysis. All data were determined on the Perkin-Elmer UV/Vis 554 Spectrophotometer with partially purified enzyme preparations (see Chapter 2). In general, the experimental results support the data presented by Sowek *et al.* [241] (Table 3.13 page 127). Figures 3.16 (page 132) and 3.17 (page 133) show the Michaelis constant and enzyme efficiency (V_{max}/K_m) data in the context of the evolutionary relationships of the TEM-*derived* β-lactamases. The K_m values appear to show that enzyme affinity for the substrate increases with additional ESBL mutations. V_{max} values, and therefore V_{max}/K_m values, for the enzymes analysed are less comparable because of the values' dependence on enzyme concentration. Maximum velocity is a linear function of turnover number (k_{cat}), therefore, when compared to other data obtained from the same enzyme preparation, it gives an indication of rate of substrate turnover. The relative V_{max} values, given in Table 3.14, infer that substrate turnover of penicillin G decreases with improvement of 3GC turnover. This trend is confirmed if the enzyme efficiency values (V_{max}/K_m) are compared.

Clavulanic acid resistant variants of the TEM β-lactamases have been described [242, 243], and have been shown to be derived from TEM-1 by one or two nucleotide mutations resulting in the substitution of amino acids at residue numbers 69 and 244 [243]. Thomson & Amyes had successfully used 'reverse' selective pressure, with ampicillin, to revert TRC-1 to TEM-1 [244]. This work demonstrated that selective pressure can reverse TEM β-lactamase mutations, even in an extreme example such as this. The clavulanic acid resistance mutation considerably reduces the MIC of a host organism to ampicillin.

		Enzyme					
		TEM-1	**TEM-5**	**TEM-9**	**TEM-10**	**TEM-12**	**TEM-26**
Plasmid		R1	pCFF14	pMG228	pJPQ100	-	pJPQ101
Amino acid:	104	Glu	Glu	LYS	Glu	Glu	LYS
	164	Arg	SER	SER	SER	SER	SER
	237	Ala	THR	Ala	Ala	Ala	Ala
	240	Glu	LYS	Glu	LYS	Glu	Glu
	265	Thr	Thr	MET	Thr	Thr	Thr
K_m (μM)	PEN G	72	56	19	65	89	36
	NCFN	7.7	43	162	31	2.8	31
	CAZ	UM	40	298	363	167	452
	CTX	UM	55	107	9.9	971	20
Relative K_m§	PEN G	935	130	11.7	210	3179	116
	NCFN	100	100	100	100	100	100
	CAZ	-	93	184	1171	5964	1458
	CTX	-	128	64	32	34679	155
V_{max} (μmol min^{-1})‡	PEN G	11	4.7	81	2.3	115	3.6
	NCFN	50	13	7.6	2.2	5.3	2.0
	CAZ	UM	8.7	24	4.3	0.24	3.4
	CTX	UM	2.5	3.1	0.06	0.13	0.06
Relative V_{max}§	PEN G	22.0	36	1066	105	2170	1.8
	NCFN	100	100	100	100	100	100
	CAZ	-	67	316	195	4.5	170
	CTX	-	19	41	2.7	2.5	3.0
V_{max}/K_m	PEN G	0.16	0.084	0.50	0.036	1.9	0.099
	NCFN	6.5	0.31	0.40	0.075	1.3	0.064
	CAZ	-	0.22	0.080	0.012	0.001	0.008
	CTX	-	0.046	0.029	0.006	0.0001	0.003
Relative V_{max}/K_m§	PEN G	2.5	27	125	48	146	155
	NCFN	100	100	100	100	100	100
	CAZ	-	71	20	16	0.08	12
	CTX	-	15	7.3	8.0	0.008	4.7

Table 3.14: Biochemical characteristics of the β-lactamases TEM-1, TEM-5, TEM-9, TEM-10, TEM-12 & TEM-26.

Abbreviations: Ala, alanine; Arg, arginine; CAZ, ceftazidime; CTX, cefotaxime; Glu, glutamic acid; Lys, lysine; Met, methionine; NCFN, nitrocefin; PEN G, penicillin G; Ser, serine; Thr, threonine; UM, unmeasurable. § All values relative to nitrocefin (100%). ‡ V_{max} values given as μmoles per minute, per ml of enzyme preparation. Amino acids annotated entirely in capital letters indicate the substituted residues in the mutant enzymes. Numbering of amino acid residues according to the recommendations of Ambler *et al.* (1991) [158]

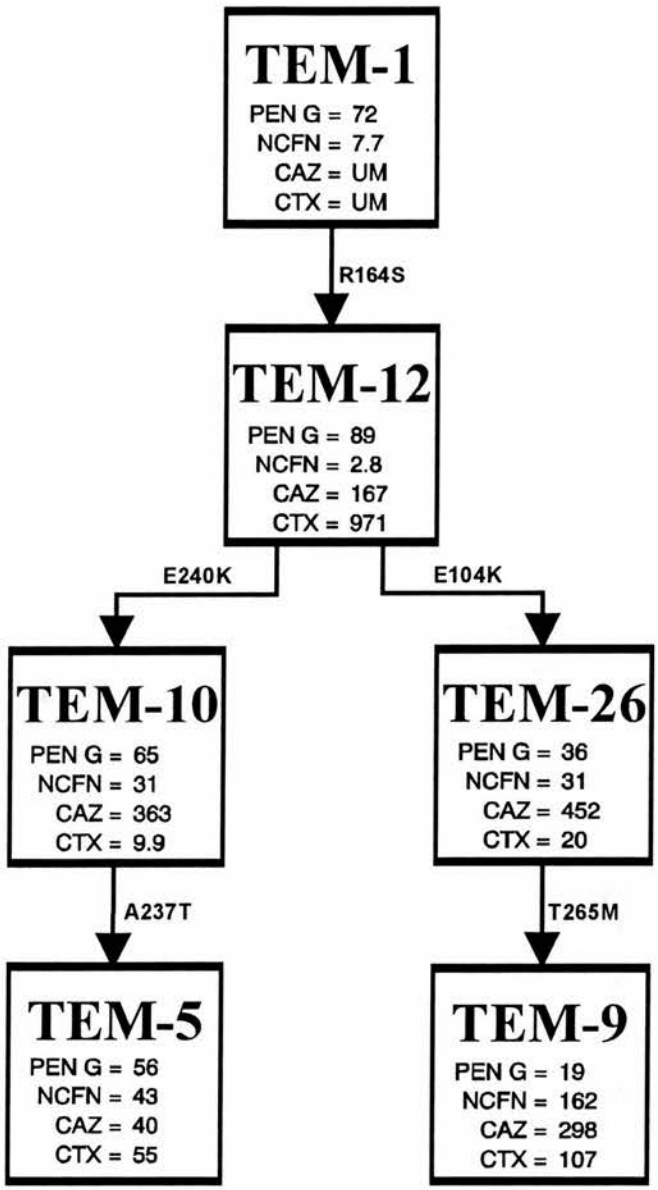

Figure 3.16: Comparison of the Michaelis constant values of the TEM-1, TEM-5, TEM-9, TEM-10, TEM-12 & TEM-26 β-lactamases.

All values of the K_m given in μmoles dm^{-3} (μM). Each arrow represents a single amino acid change facilitated by a single nucleotide change in the β-lactamase gene. One letter amino acid notation according to the IUPAC-IUB Commission on Nomenclature [237]: A, alanine; E, glutamic acid; K, lysine; M, methionine; R, arginine; S, serine; T, threonine. Numbering of amino acid residues according to the recommendations of Ambler *et al.* (1991) [158]. Abbreviations: CAZ, ceftazidime; CTX, cefotaxime; NCFN, nitrocefin; PEN G, penicillin G; UM, unmeasurable.

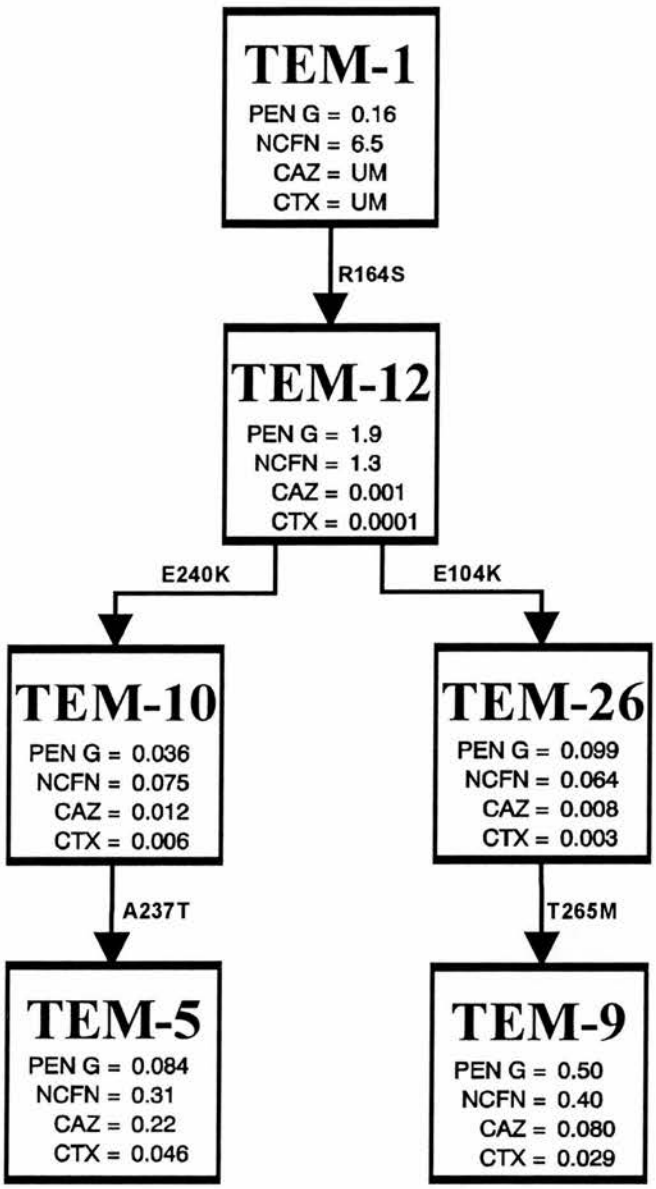

Figure 3.17: Comparison of the enzyme efficiency of the TEM-1, TEM-5, TEM-9, TEM-10, TEM-12 & TEM-26 β-lactamases.

All values of the enzyme efficiency, V_{max}/K_m, given in dm^3 $minute^{-1}$ per ml of enzyme preparation. Each arrow represents a single amino acid change facilitated by a single nucleotide change in the β–lactamase gene. One letter amino acid notation according to the IUPAC-IUB Commission on Nomenclature [237]: A, alanine; E, glutamic acid; K, lysine; M, methionine; R, arginine; S, serine; T, threonine. Numbering of amino acid residues according to the recommendations of Ambler *et al.* (1991) [158]. Abbreviations: CAZ, ceftazidime; CTX, cefotaxime; NCFN, nitrocefin; PEN G, penicillin G; UM, unmeasurable.

7.2. APPLICATION OF SELECTIVE PRESSURE WITH AMPICILLIN

From the analysis of the data obtained from the results of Sowek *et al.* [241], my own enzyme kinetic analysis and the results of Thomson & Amyes [244], the application of 'reverse' selective pressure with a broad-spectrum penicillin would theoretically promote back-mutations of an ESBL in a suitable host organism.

Employing a method for the continuous sub-culturing of a TEM β-lactamase expressing *E. coli*, based upon that described by Thomson & Amyes [244] a strategy was designed for a passage experiment. *E. coli* J62-2 harbouring the 141-kb clinical plasmid pCFF14 encoding the TEM-5 β-lactamase was the ESBL

Enzyme	Plasmid	*E. coli* Strain	MIC (mg/l)§				
			AMP	AMX	AMX/CLAV	CAZ	CTX
TEM-1	R1	J62-2	2048	1024	8	0.125	0.0313
TEM-12	-	MG32	3072	3072	16	8	0.25
TEM-10	pJPQ100	J53-2	4096	4096	16	64	0.5
TEM-5	pCFF14	J62-2	1024	1024	8	16*	1
TEM-3	pCFF04	J62-2	2048	-	-	64	8‡
TEM-6	pMG226	J62-2	2048	-	-	64	1‡
TEM-9	pMG228	J62-2	2048	-	-	128	2‡
TEM-E2	pUK721	J53-2	5120	3072	16	16	0.25

Table 3.15: Antibiotic susceptibility of selected TEM β-lactamases.

Abbreviations: AMP, ampicillin; AMX, amoxycillin; AMX/CLAV, amoxycillin/clavulanic acid (ratio 2:1); CAZ, ceftazidime; CTX, cefotaxime. § Inoculum of 10^4 cfu/spot for each test organism. * The MIC values determined against *E. coli* J62-2 (pCFF14) throughout this work were consisitently atypical compared with other workers results (usually the MIC of CAZ = 64mg/l [184]), for the purposes of the Discussion (Chapter 4) the higher value is assummed to be the correct value. ‡ The MIC values of cefotaxime were not determined but the values given are taken from published sources for the purposes of comparison: TEM-3 [183], TEM-6 [185], and TEM-9 [187].

chosen for use in the experiment.

The antibiotic susceptibility of host organisms expressing the TEM-1 and TEM-5 β-lactamase and the intermediate enzymes TEM-10 and TEM-12 were determined together with a number of other ESBL expressing organisms (Table 3.15). MICs were used to ascertain variations in levels of resistance to penicillins and the 3GCs together with penicillin/β-lactamase inhibitor combinations. The antibiotic susceptibilities show that ceftazidime and cefotaxime MICs increase in the series TEM-1→TEM-12→TEM-10→TEM-5. No similar trend was observed for the susceptibility to ampicillin, amoxycillin or amoxycillin/clavulanic acid.

PASSAGE EXPERIMENT METHODOLOGY

The passage experiment methodology was a modification of that described by Thomson & Amyes [244]: *E. coli* J62-2 (TEM-5_{pCFF14}) was cultured in three separate 100ml aliquots of liquid media (IST broth) containing no antibiotic (0mg/l),1024mg/l ampicillin and 2048mg/l ampicillin respectively. The cultures were incubated overnight at 37°C in an orbital shaker. After incubation 100µl of each was subcultured into a freshly prepared 100ml aliquot of liquid media containing the same antibiotic concentration (i.e. containing 0mg/l, 1024mg/l and 2048mg/l ampicillin respectively). The overnight cultures were subcultured into fresh media a total of five times (five days). Twelve colonies were purified from each subculture on each day of the passage.

The MICs of ampicillin, ceftazidime and cefotaxime were ascertained according to the BSAC guidelines [196] as described in Chapter 2.

IEF, employing broad-range carrier ampholytes (pH 3.5-10), was used to examine a selection of the β-lactamases produced.

AMPICILLIN PASSAGE EXPERIMENT RESULTS

One hundred & eighty strains were purified from the ampicillin passage experiment. Each strain was checked for lactose utilisation, rifampicin resistance and histidine auxotrophy to confirm that each strain was *E. coli* J62-2. One hundred of the 180 purified strains were tested for antibiotic susceptibilities together with strains expressing TEM-1_{R1}, TEM-12, TEM-$10_{pJPQ100}$, and

TEM-5_{pCFF14}. A summary of the MIC results are given in Table 3.16 (page 137). Figure 3.18 (page 138) shows a plot of average MIC of the purified colonies against the day of isolation, to demonstrate the drift of both ampicillin and ceftazidime resistance under selective pressure. Under no selective pressure the MICs of both ampicillin and ceftazidime remained constant, no decrease was observed. Both the 1024mg/l ampicillin and 2048mg/l ampicillin passage experiments show a general increase of the MICs of both ampicillin and ceftazidime over the five days.

A number of the strains for which the ampicillin and ceftazidime MICs had been determined were selected for more detailed examination. MICs of ampicillin, amoxycillin, amoxycillin/clavulanic acid (ratio 2:1), ceftazidime and cefotaxime were then determined (see Table 3.17 page 139). The isoelectric points of the enzymes produced by the nine strains were identified from crude β-lactamase preparations. All strains were found to produce only a single enzyme of pI 5.5 that aligned perfectly with the single focused band of TEM-5_{pCFF14} (Table 3.17). The isoelectric points of TEM-5 and the nine strains were different from that of TEM-$10_{pJPQ100}$ (pI 5.57), TEM-12 (pI 5.25) and TEM-1_{R1} (pI 5.4).

CONCLUDING REMARKS

No β-lactamase mutations were observed to have been selected by the ampicillin passage experiment on the basis of isoelectric points and antibiotic susceptibility profiles. The increase in the MIC of the β-lactam antibiotics tested for the strains examined were presumed to have resulted in reduced penetration of the drugs into the cell because of OMP changes. The OMPs of the purified strains were not examined.

Ampicillin Concentration‡	No. of strains	MIC (mg/l)§	
		AMP	CAZ
0mg/l	30	<1000	16
1024mg/l	1	<1000	64
	7	1250	64
	26	1500	64
	1	2000	128
2048mg/l	5	1500	64
	1	1500	128
	9	1750	128
	3	2000	128
	2	3000	128
	1	3250	64
	2	3250	128
	1	3250	>128
	8	3500	64
	2	3500	128
	1	3500	>128

Table 3.16: Changes in ampicillin & ceftazidime MICs observed in the ampicillin passage experiment.

Abbreviations: AMP, ampicillin; CAZ, ceftazidime. ‡ Concentration of antibiotic used in passage experiment. § Inoculum of 10^4 cfu/spot for each test organism.

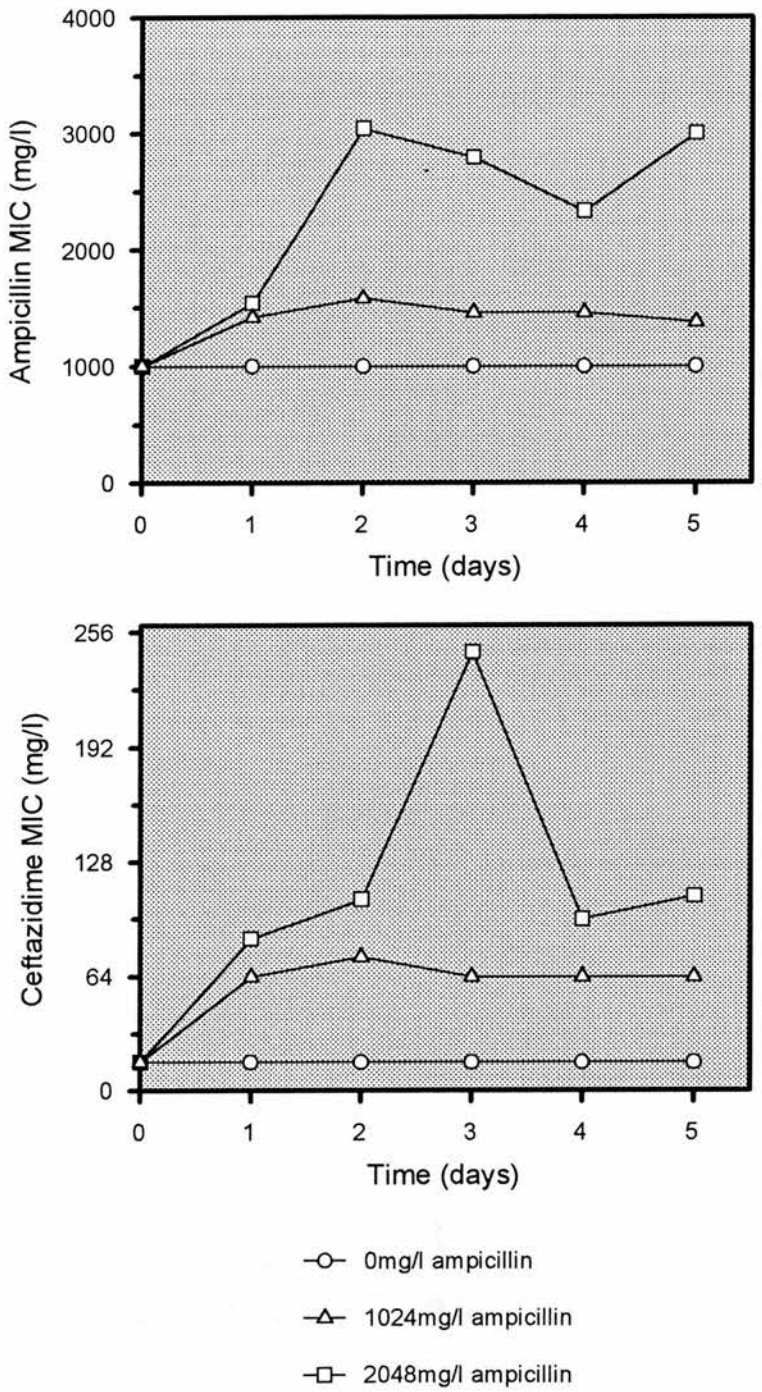

Figure 3.18: Changes in ampicillin & ceftazidime MICs observed in the ampicillin passage experiment.

The mean MIC (mg/l) of ampicillin and ceftazidime for the purified colonies isolated was plotted on a daily basis from the ampicillin passage experiment. Time expressed in days from first inoculation.

Antibiotic conc.‡	Day	Strain No.	pI	MIC (mg/l)§				
				AMP	AMX	AMX/ CLAV	CAZ	CTX
0mg/l	3	0/27	5.5	512	1024	8	8	1
1024mg/l	1	1024/11	5.5	2048	2048	16	>64	4
	2	1024/23	5.5	2048	2048	8	>64	4
	3	1024/27	5.5	2048	2048	16	>64	4
2048mg/l	2	2048/25	5.5	3072	3072	16	>64	8
	3	2048/36	5.5	3072	3072	16	>64	8
	4	2048/41	5.5	3072	3072	16	>64	8
	5	2048/55	5.5	4096	3072	16	64	8
	5	2048/57	5.5	2048	3072	16	>64	8
Controls:								
TEM-1_{R1}			5.4	2048	1024	8	0.125	0.0313
TEM-12			5.25	3072	3072	16	8	0.25
TEM-$10_{pJPQ100}$			5.57	4096	6144	16	>64	0.5
TEM-5_{pCFF14}			5.5	512	1024	8	16	1

Table 3.17: MIC profiles of nine ampicillin passage experiment strains.

‡ Concentration of antibiotic used in passage experiment. § Inoculum of 10^4 cfu/spot for each test organism. Abbreviations: AMP, ampicillin; AMX, amoxycillin; AMX/CLAV, amoxycillin/clavulanic acid (ratio 2:1); CAZ, ceftazidime; CTX, cefotaxime; pI, isoelectric point.

7.3. THE EFFECT OF CLAVULANIC ACID ON THE ESBL.

The effect of ampicillin upon the ESBL TEM-5 did not promote back mutations as expected from the study of the enzyme kinetic data. Comparison of the concentrations of the β-lactamase inhibitor clavulanic acid required to inhibit 50% of enzyme activity, I_{50} values, (with cephaloridine as the substrate) [241], demonstrated a rise in susceptibility proportional to the number of amino acid substitutions present in each ESBL (see Table 3.13 page 127). Study of the I_{50} values of clavulanic acid for the enzymes TEM-1, TEM-5, TEM-9, TEM-10, TEM-12, and TEM-26, in the presence of penicillin G, revealed a similar trend (see Table 3.18 page 141 and Figure 3.19 page 142). I_{50} values for clavulanic acid were determined by incubating the enzyme and inhibitor for 5 minutes at 37°C before the reaction was initiated by the addition of penicillin G to a final concentration of 1mM. Successive sub-cultures of an ESBL expressing organism in the presence of clavulanic acid and amoxycillin were then investigated. The selective pressure exerted by a competitive and progressive β-lactamase inhibitor, such as clavulanic acid, when combined with a broad-spectrum penicillin was suspected to be far greater than that exerted by a broad-spectrum penicillin alone.

PASSAGE EXPERIMENT METHODOLOGY

The methodology employed was the same as that described for the ampicillin passage experiment, except for the application of selective pressure with amoxycillin in combination with clavulanic acid (2:1 ratio). *E. coli* J62-2 ($TEM\text{-}5_{pCFF14}$) was cultured in liquid media at four different amoxycillin/clavulanic acid (co-amoxiclav) concentrations: no antibiotic (0mg/l), 4mg/l co-amoxiclav, 8mg/l co-amoxiclav and 16mg/l co-amoxiclav respectively. All concentrations of co-amoxiclav were expressed in terms of the amoxycillin concentration within the combination. Cultures were subcultured into liquid media containing the same antibiotic concentration, if bacterial growth was observed. The overnight cultures were subcultured into fresh media five times after incubation at 37°C. Ten colonies were purified from each subculture on each day of the passage.

		Enzyme					
		TEM-1	TEM-5	TEM-9	TEM-10	TEM-12	TEM-26
Plasmid		R1	pCFF14	pMG228	pJPQ100	-	pJPQ101
Amino acid:	104	Glu	Glu	LYS	Glu	Glu	LYS
	164	Arg	SER	SER	SER	SER	SER
	237	Ala	THR	Ala	Ala	Ala	Ala
	240	Glu	LYS	Glu	LYS	Glu	Glu
	265	Thr	Thr	MET	Thr	Thr	Thr
I_{50} (μM)‡: Clav/1mM Pen G		3.6	1.2	0.38	2.3	2.9	1.1

Table 3.18: I_{50} values for clavulanic acid of the selected TEM β-lactamases.

Abbreviations: Ala, alanine; Arg, arginine; Clav, clavulanic acid; Glu, glutamic acid; Lys, lysine; Met, methionine; PEN G, penicillin G; Ser, serine; Thr, threonine. ‡ Enzyme and inhibitor incubated for 5 minutes at 37°C before the reaction was initiated by the addition of penicillin G to 1mM. Amino acids annotated entirely in capital letters indicate the substituted residues in the mutant enzymes. Numbering of amino acid residues according to the recommendations of Ambler *et al.* (1991) [158]

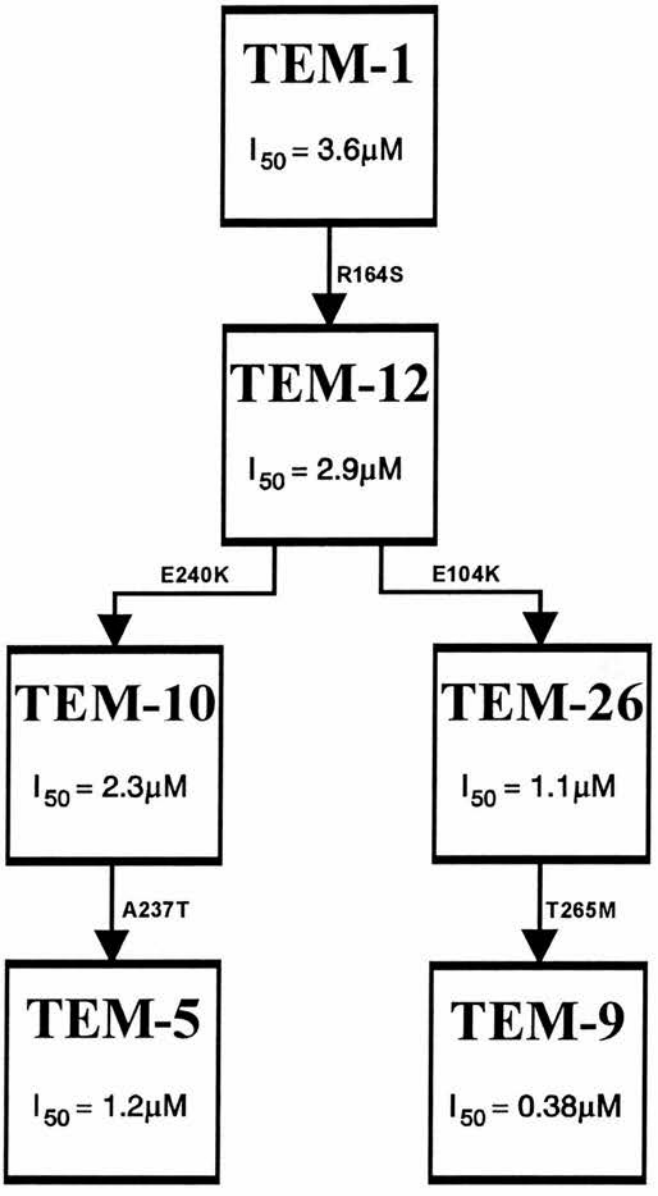

Figure 3.19: Comparison of the I_{50} values for clavulanic acid of the TEM-1, TEM-5, TEM-9, TEM-10, TEM-12 & TEM-26 β-lactamases.

All I_{50} values are given in μmoles dm^{-3} (μM) and were measured by incubating the enzyme and various concentrations of clavulanic acid for 5 minutes at 37°C, before adding penicillin G (to 1mM). Each arrow represents a single amino acid change facilitated by a single nucleotide change in the β–lactamase gene. One letter amino acid notation according to the IUPAC-IUB Commission on Nomenclature [237]: A, alanine; E, glutamic acid; K, lysine; M, methionine; R, arginine; S, serine; T, threonine. Numbering of amino acid residues according to the recommendations of Ambler *et al.* (1991) [158].

MICs of ampicillin, amoxycillin, ceftazidime, cefotaxime and amoxycillin/clavulanic acid were ascertained. IEF employing a broad-range mixture of carrier ampholytes (pH 3.5-10 and pH 4-6) was used to examine a selection of the β-lactamases produced. β-Lactamase activity and the effect of clavulanic acid inhibition were determined by spectrophotometric assays.

The method of Takahashi & Nagano [213] (described in Chapter 2) was used to extract and visualise the plasmid DNA of those strains examined by IEF. The plasmid DNA was also digested with a number of restriction endonucleases, none of which cut within the TEM β-lactamase ORF. Southern blots were prepared and were subsequently analysed by hybridisation with a non-radioactively (biotin) labelled *bla*T-1_{pAT153} probe.

CO-AMOXICLAV PASSAGE RESULTS

The inoculum in IST broth containing 16mg/l co-amoxiclav failed to grow after the first overnight incubation and was therefore discontinued. One hundred & fifty strains were purified from the ampicillin passage experiment. Each strain was checked for lactose utilisation, rifampicin resistance, and histidine auxotrophy to confirm that each strain was an *E. coli* J62-2. Sixty-seven of the 150 purified strains (15 from the 0mg/l co-amoxiclav passage, 25 from the 4mg/l co-amoxiclav passage and 27 from the 8mg/l co-amoxiclav passage) were tested for susceptibility to ampicillin, amoxycillin, co-amoxiclav, ceftazidime and cefotaxime, together with strains expressing TEM-1_{R1}, TEM-12, TEM-$10_{pJPQ100}$, and TEM-5_{pCFF14}. A summary of the MIC results is given in Table 3.19 (page 144) together with graphical representations of the observed change in antibiotic susceptibilities in Figures 3.20, 3.21 & 3.22 (pages 145-147).

A selection of the strains identified with anomalous antibiotic susceptibilities, together with a selection of strains with 'modal' MIC results from each of the three antibiotic concentration groups of the passage experiment, were analysed by IEF to identify any abnormalities in β-lactamase band focusing.

Co-amoxiclav Conc.‡	No. of strains	MIC (mg/l)§				
		AMP	AMX	AMX/CLAV	CAZ	CTX
0mg/l	14	<256	1024	8	8	0.5
	1	<256	1024	8	16	0.5
4mg/l	1	<256	1024	4	16	0.5
	4	<256	1024	8	16	0.5
	4	512	1024	8	16	1
	12	512	2048	8	32	0.5
	1	512	2048	8	32	1
	2	1024	2048	8	32	1
	1†	2048	2048	8	64	2
8mg/l	15	512	2048	16	32	4
	1	1024	2048	16	64	2
	1	1024	2048	16	64	4
	2	1024	2048	16	64	8
	1	1024	2048	16	128	4
	4	2048	2048	16	128	8
	2	2048	3072	16	128	16
	1*	3072	3072	4	>128	8
Controls:						
$TEM\text{-}1_{R1}$		1024	2048	8	<0.0625	0.006
TEM-12		5120	4096	16	16	0.125
$TEM\text{-}10_{pJPQ100}$		3072	5120	8	64	0.5
$TEM\text{-}5_{pCFF14}$		512	2048	8	16	0.5

Table 3.19: Changes in antibiotic susceptibilities observed in the co-amoxiclav passage experiment.

‡ Concentration of antibiotic used in passage experiment. § Inoculum of 10^4 cfu/spot for each test organism. † *E. coli* J62-2 strain no. 4/10. * *E. coli* J62-2 strain no. 8/8. Abbreviations: AMP, ampicillin; AMX, amoxycillin; AMX/CLAV, amoxycillin/clavulanic acid (ratio 2:1); CAZ, ceftazidime; CTX, cefotaxime.

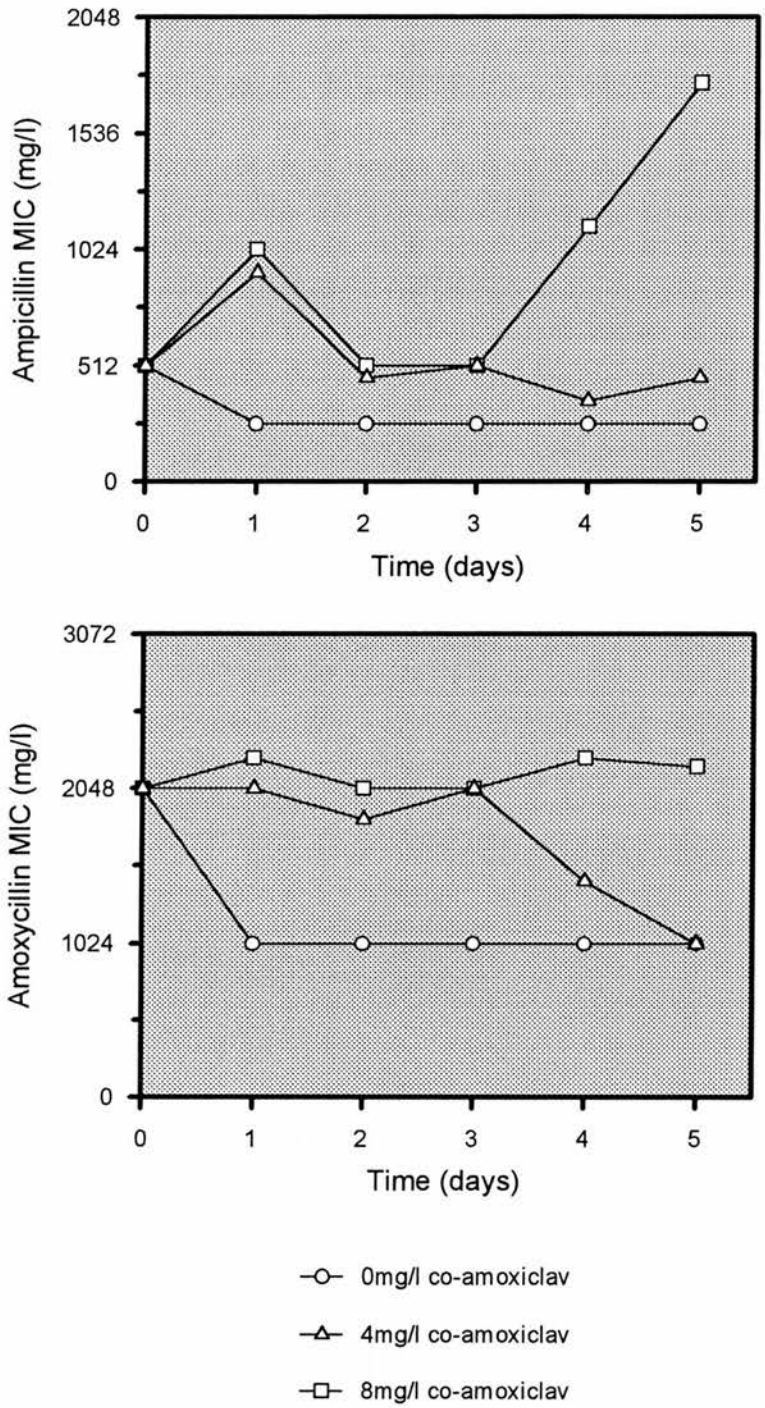

Figure 3.20: Changes in ampicillin & amoxycillin MICs observed in the co-amoxiclav passage experiment.

The mean MIC (mg/l) of ampicillin and amoxycillin for the purified colonies isolated plotted on a daily basis from the co-amoxiclav passage experiment. Time expressed in days from first inoculation.

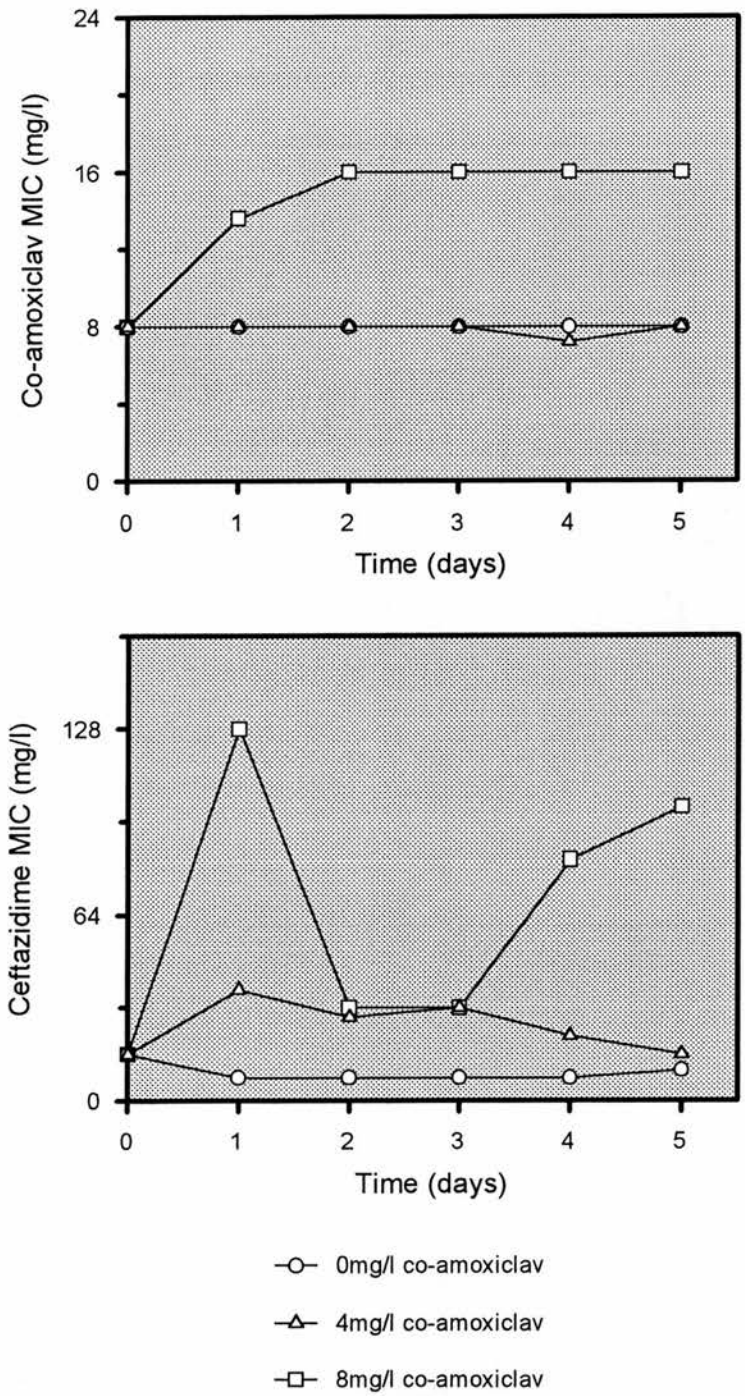

Figure 3.21: Changes in co-amoxiclav & ceftazidime MICs observed in the co-amoxiclav passage experiment.

The mean MIC (mg/l) of co-amoxiclav and ceftazidime for the purified colonies isolated plotted on a daily basis from the co-amoxiclav passage experiment. Time expressed in days from first inoculation.

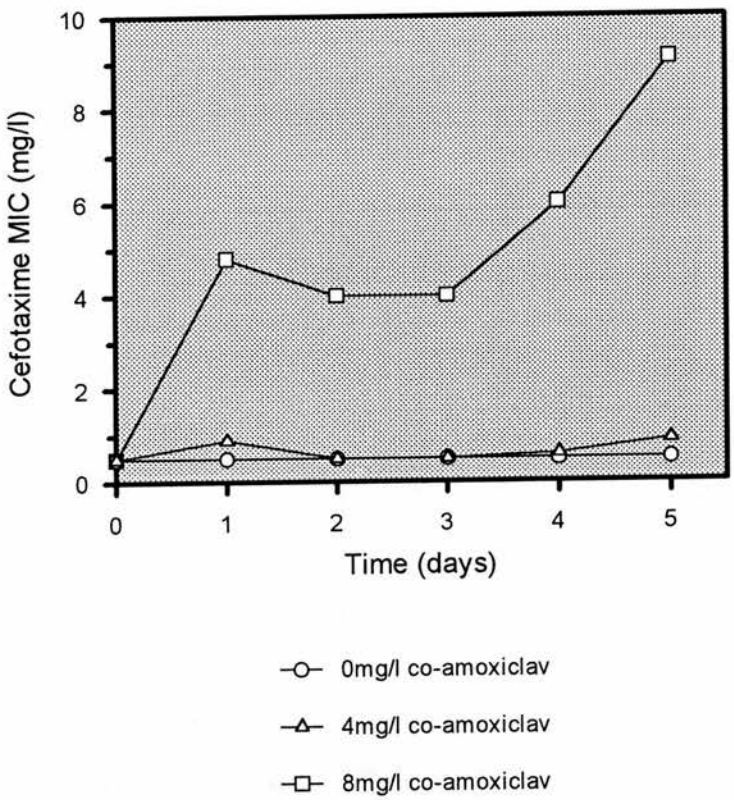

Figure 3.22: Changes in cefotaxime MIC observed in the co-amoxiclav passage experiment.

The mean MIC (mg/l) of cefotaxime for the purified colonies isolated plotted on a daily basis from the co-amoxiclav passage experiment. Time expressed in days from first inoculation.

Some of the nine strains examined demonstrated multiple bands of β-lactamase activity after visualisation of the IEF gel, compared with the single band (pI 5.5) obtained from a preparation of TEM-5_{pCFF14} (Figure 3.23 page 149). Two strains (numbers 4/10 and 8/8) were selected for more detailed analysis: each of the two strains exhibited two bands in addition to a band that focused at exactly the same point as that of TEM-5_{pCFF14}; one of pI 5.25 and one of pI 5.57. These two additional bands focused at the same isoelectric point as the focused bands of TEM-10_{pCFF14} (pI 5.57) and TEM-12 (pI 5.25). Both strains exhibited strong pI 5.5 and 5.25 bands, however, strain number 8/8 exhibited a strong pI 5.57 band and strain number 4/10 showed a weak pI 5.57 band.

The graphs obtained from the results of the co-amoxiclav passage experiment (see Figures 3.20, 3.21 & 3.22) show that the MICs of the antibiotics tested changed in a similar manner to that observed during the ampicillin passage experiment. In general, the antibiotic susceptibilities increased with time after challenge with either 4mg/l or 8mg/l co-amoxiclav. The control experiment (0mg/l co-amoxiclav) did not raise the MIC of any of the antibiotics tested.

ANALYSIS OF *E. COLI* J62-2 STRAIN NUMBERS 4/10 & 8/8

The plasmid DNA from strain number 4/10 and strain number 8/8 was isolated and visualised by agarose gel electrophoresis, and compared with plasmid DNA from the parent strain, *E. coli* J62-2 (pCFF14). Each of the strains examined was shown to contain a single plasmid ~141-kb in size. The plasmid DNA of strain numbers 4/10 and 8/8 was then compared to pCFF14 by restriction analysis (Figure 3.24 page 151) with a variety of restriction endonucleases that did not have recognition sites within the *bla*T gene. Plasmid DNA from strain numbers 4/10 and 8/8 were found to have identical restriction patterns for all the restriction endonucleases used in the analysis. Comparison of the restriction patterns of pCFF14 with that of the plasmids isolated from the mutant strains revealed an additional *Eco*R I restriction fragment, about 25-30-kb in size. Additional bands were also observed when the plasmid DNA of strain numbers 4/10 and 8/8 were restricted with other restriction endonucleases. Apart from the additional bands, restriction patterns of pCFF14 and the mutant plasmids were identical.

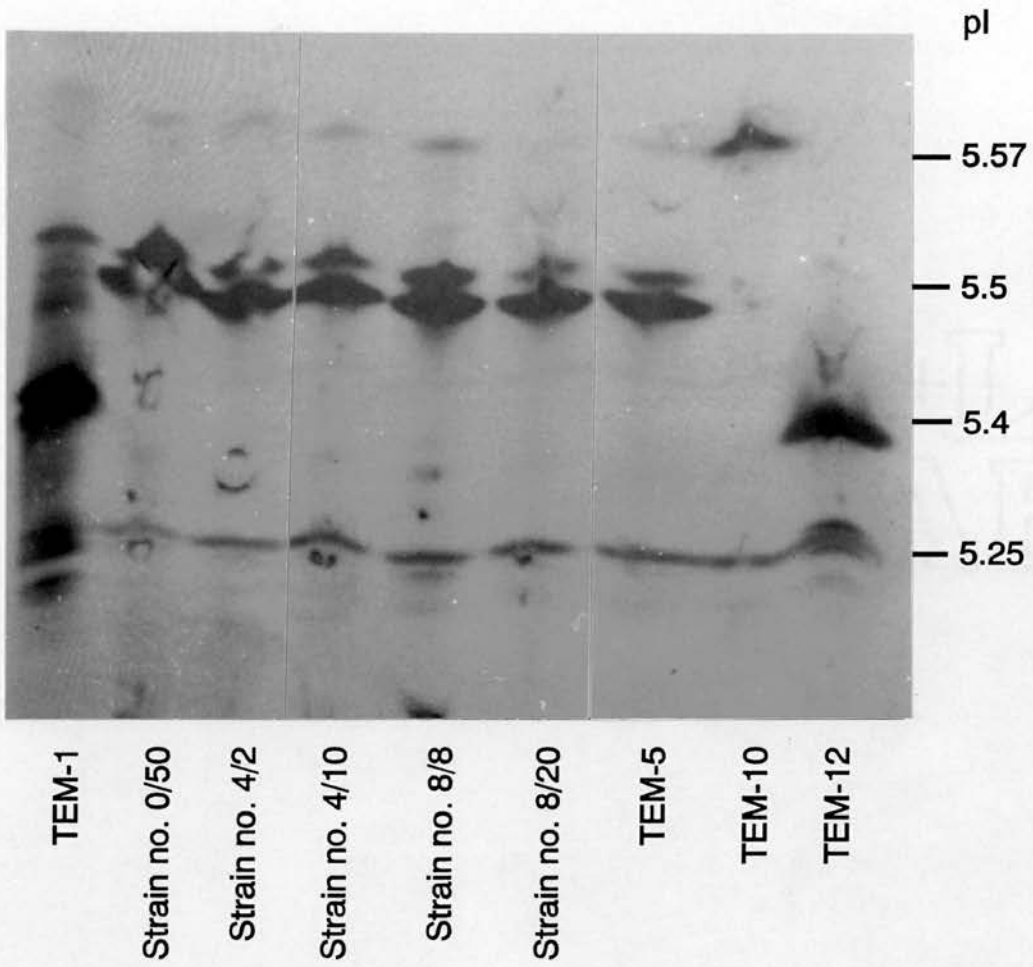

Figure 3.23: IEF gel showing the TEM-*type* β-lactamases from co-amoxiclav passage experiment.

The IEF gel contained carrier ampholytes pH range 4-6. Crude extracts of test organisms were prepared, and isoelectric points of the enzymes produced by each strain determined by comparison with preparations of enzymes of known pI. Standard β-lactamases shown include TEM-1 (pI 5.4), TEM-5 (pI 5.5), TEM-10 (pI 5.57), and TEM-12 (pI 5.25). The TEM-12 preparation also exhibits a TEM-1 band. The pI 5.25 bands present in the enzyme preparations in this figure were shown to be satellite bands by repeated IEF experiments except in the TEM-12 preparation and the extracts of strain numbers 4/10 and 8/8.

The results of the plasmid DNA analysis suggested that the mutant plasmid was closely related to pCFF14 and was designated pUK3007.

Southern blotting and hybridisation (Figure 3.25 page 152) of the restricted DNA fragments using a 900-bp biotin-labelled probe generated by PCR from *bla*T-1_{pAT153}, revealed that pCFF14 encoded a single TEM β-lactamase gene; however, pUK3007 encoded two TEM β-lactamase genes. One of the *bla*T genes of pUK3007 was always observed on the same restriction fragment as the single *bla*T-5 gene of pCFF14. The second *bla*T gene from pUK3007 was present on the additional 25-30-kb restriction fragment of the *Eco*R I digest. The second *bla*T gene of pUK3007 was always found to be present on one of the additional bands from the other restriction digests.

KINETIC ANALYSIS OF MUTANT β-LACTAMASES

The kinetic profiles of the mutant strains' β-lactamases were studied with the Perkin-Elmer UV/Vis 554 Spectrophotometer. The K_m and V_{max} values of the partially purified mixed enzyme preparations were determined for the substrates penicillin G, nitrocefin, ceftazidime and cefotaxime and the I_{50} value for clavulanic acid in the presence of penicillin G. The three enzymes from partially purified preparations of *E. coli* J62-2 strain number 8/8 were separated by preparative IEF.

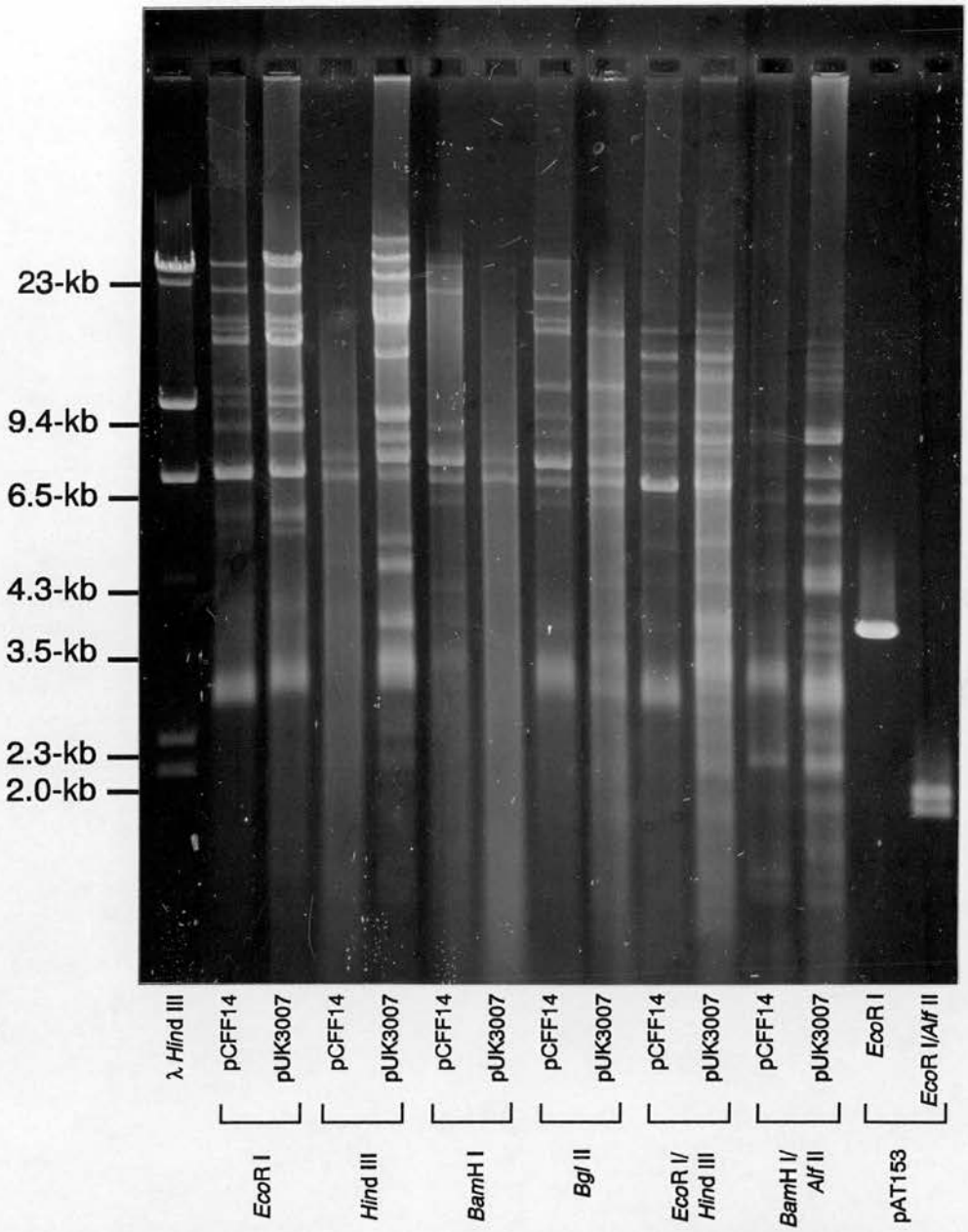

Figure 3.24: Restriction analysis comparing pCFF14 & pUK3007

The parent plasmid pCFF14 (141-kb) and the mutant plasmid pUK3007 (~170-kb) were digested with the restriction endonucleases *Alf* II, *Bam*H I, *Bgl* II, *Eco*R I and *Hind* III. λ DNA digested with *Hind* III was used as molecular weight markers. pAT153 was digested with *Eco*R I and *Eco*R I/*Alf* II as a control restriction experiments. Identical mutant plasmids, designated pUK3007, were isolated from strain numbers 4/10 and 8/8. The pCFF14/*Eco*R I digest was compared to that published by Chanal *et al.*(1988)[184] and was identical.

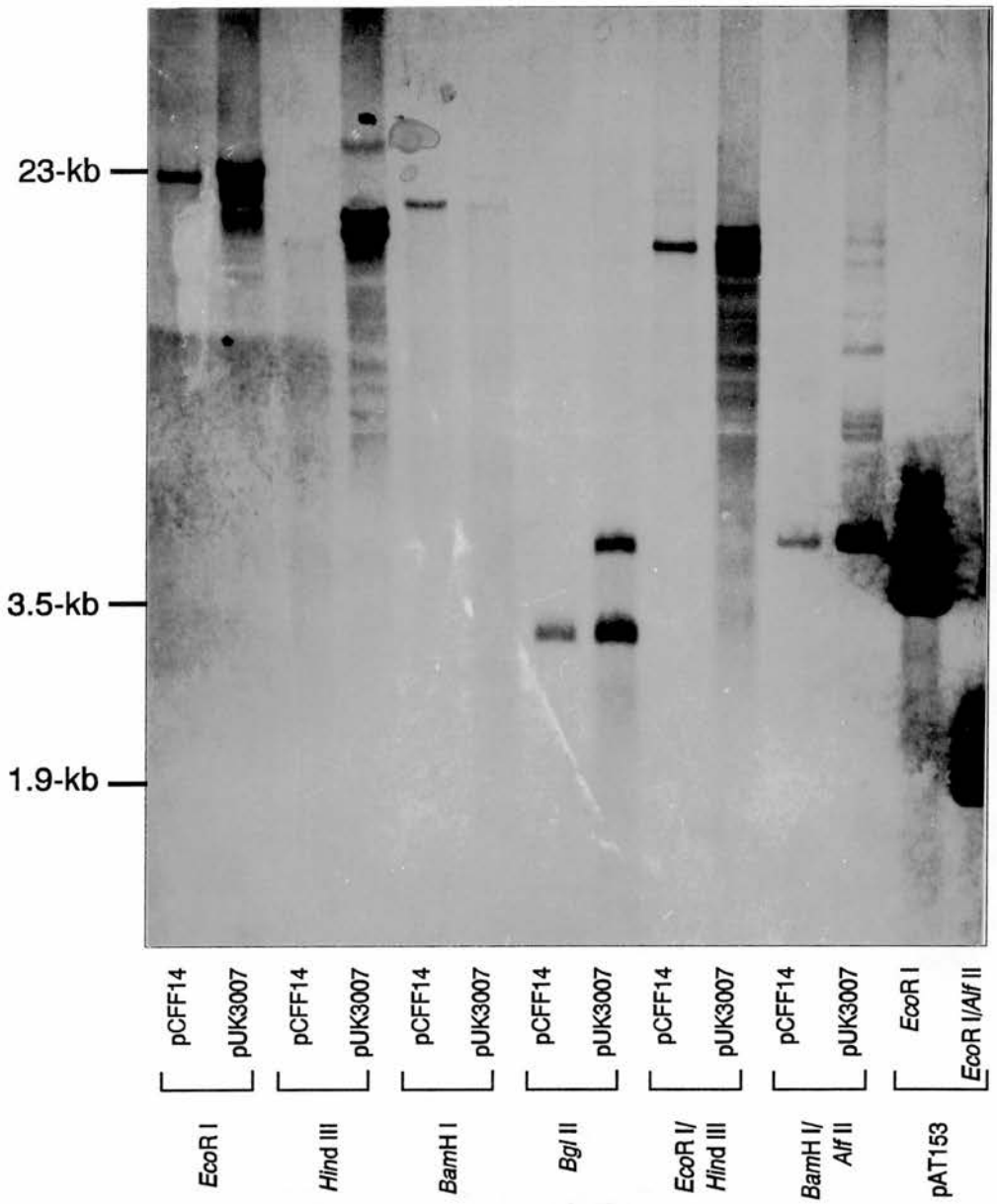

Figure 3.25: Southern blot of the restriction analysis comparing pCFF14 & pUK3007

Southern transfer of restriction endonuclease digested plasmid DNA (see Figure 3.24 page 151) was probed with a PCR-generated biotin labelled 900-bp *bla*T-1$_{pAT153}$ fragment to reveal the DNA fragments encoding TEM β-lactamases genes. Digested pAT153 DNA as used as a positive control.

The three separated enzyme bands (pI 5.57, 5.5, and 5.25) were then used to determine the kinetic profiles of each of the enzymes for the substrates benzylpenicillin, nitrocefin, ceftazidime and cefotaxime, together with the concentration of clavulanic acid required to inhibit 50% of enzyme activity with penicillin G as the substrate. A summary of the kinetic analysis to the collective enzymes and the individual enzymes is given in Table 3.20 (page 155). Each of the separated enzyme preparations show kinetics comparable with known β-lactamases. These results suggest that the three enzymes encoded by strain numbers 4/10 and 8/8 were TEM-12, TEM-5 and TEM-10 respectively. The K_m of the pI 5.25 enzyme for the substrate cefotaxime was unmeasurable because of the exceptionally low V_{max} value obtained from the pure preparation. The partially pure preparation of the pUK3007 β-lactamases (containing the three enzymes) possessed a much broader spectrum of activity than any of the individual enzymes. The I_{50} value for the combined pUK3007 enzymes (2.2μM) is approximately equivalent to the mean of the I_{50} values for clavulanic acid of the three separated β-lactamases (2.8μM).

DIRECT DNA SEQUENCING OF PCR-AMPLIFIED DNA WITH DYNABEADS

The method developed for the sequencing of the *bla*T genes of pUK721 (see under Section 5.6 page 120) was employed to obtain DNA sequences from the pUK3007 encoded *bla*T genes. The target DNA was amplified by the PCR reaction with one biotinylated PCR-primer (Biotin-Bla-4') and one non-labelled PCR primer (Bla-3'). The double-stranded PCR-product was immobilised on Dynabeads M-280 Streptavidin and the non-biotinylated amplimer was eluted to provide a single-stranded DNA sequencing template. The template DNA from a single PCR reaction was re-used after salvage from each sequencing reaction mixture prior to loading onto the polyacrylamide gel for electrophoresis. Re-use of the template required stringent denaturation and washing steps to remove all of the radiolabelled sequencing reaction products. Sequencing reaction products were separated by polyacrylamide gel electrophoresis, and the dried gels autoradiographed for ~12 hours. From the sequences generated from pUK3007 samples by this method, two duplicate bands were observed (Figure 3.26 page 156):

a) Two nucleotide bands were observed at position 1001 corresponding to both guanine and adenine.

b) Two nucleotide bands, guanine and adenine, were present at position 1007.

This shows that the codons for Ala-237 (GCC), Thr-237 (ACC), Glu-240 (GAG) and Lys-240 (AAG) were present in the PCR-DNA. No other duplicate bands were observed at positions simultaneous to other nucleotide bands at other points in the PCR-generated *bla*T genes from pUK3007. Simultaneous bands resulting from leakage of reaction mixes between lanes of the sequencing gel were, therefore, not a possibility. Control reactions using PCR-DNA generated from pBR322 exhibited no such duplicate bands over the same nucleotide sequence regions.

This experiment has shown that the *bla*T genes encoded by the pUK3007 plasmid(s) encode all the nucleotide changes observed in the TEM-12→ TEM-10→TEM-5 β-lactamase series. The presence of the wild-type (TEM-5) genotype, together with the TEM-12 and TEM-10 genotypes, confirm the observations from the IEF and kinetic analyses that the three β-lactamase bands signify the presence of TEM-12, TEM-10 and TEM-5 in *E. coli* J62-2 Strain numbers 4/10 and 8/8.

		Enzyme(s)			
		Unknown	**Unknown**	**Unknown**	**Unknown**
Isoelectric point(s)		5.25 5.5 5.57	5.25	5.5	5.57
Source plasmid:		pUK3007	pUK3007	pUK3007	pUK3007
K_m (μM)	PEN G	45	182	314	189
	NCFN	26	43	12	20
	CAZ	491	281	80	359
	CTX	37	UM	28	19
Relative K_m§	PEN G	173	423	2600	945
	NCFN	100	100	100	100
	CAZ	1888	653	669	1795
	CTX	142	-	232	95
V_{max} (μmol min^{-1})*	PEN G	7.4	0.21	0.53	0.55
	NCFN	19	2.2	0.92	0.61
	CAZ	17	0.33	0.50	0.37
	CTX	2.8	0.066	0.39	0.08
Relative V_{max}§	PEN G	39	9.5	58	90
	NCFN	100	100	100	100
	CAZ	89	15	54	61
	CTX	15	3.0	42	13
V_{max}/K_m	PEN G	0.16	0.001	0.002	0.003
	NCFN	0.74	0.05	0.08	0.03
	CAZ	0.04	0.001	0.006	0.01
	CTX	0.07	-	0.01	0.004
Relative V_{max}/K_m§	PEN G	22	20	2.6	9.6
	NCFN	100	100	100	100
	CAZ	5.4	20	7.7	33
	CTX	9.5	-	17	13
I_{50} (μM)‡:	Clav	2.2	4.5	1.9	2.0

Table 3.20: Enzyme kinetics of the β-lactamases encoded by pUK3007.

§ All values relative to nitrocefin (100%). * V_{max} values given as μmoles per minute, per ml of enzyme preparation. ‡ Enzyme and inhibitor (clavulanic acid) incubated for 5 minutes at 37°C before the reaction was initiated by the addition of penicillin G to 1mM. Abbreviations: CAZ, ceftazidime; Clav, clavulanic acid; CTX, cefotaxime; NCFN, nitrocefin; PEN G, penicillin G; UM, unmeasurable.

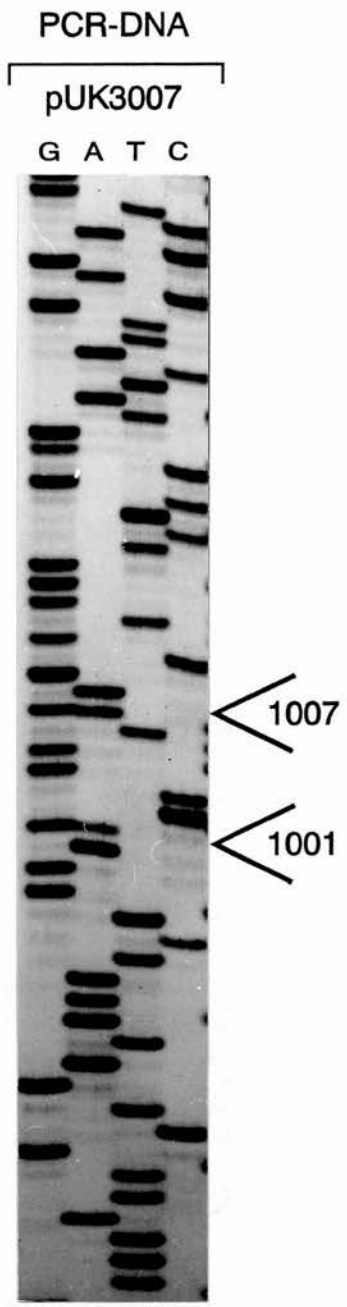

Figure 3.26: Results of the direct sequencing of PCR-products from pUK3007 using Dynabeads

The photographs of the autoradiographs are marked to show nucleotide position 1001 and 1007. PCR-amplified DNA from pUK3007 demonstrated simultaneous bands (guanine & adenine) at nucleotide position 1001 and 1007.

7.4. THE EFFECT OF FURTHER "REVERSE" SELECTIVE PRESSURE ON *E. COLI* J62-2 (pUK3007).

The back-mutation of the TEM-5 ESBL to the intermediate ESBL, TEM-10 and TEM-12, suggested the following hypothesis: continued sub-culture of the pUK3007 harbouring strains under the selective pressure of co-amoxiclav would select out plasmids encoding *bla*T-1 in preference to plasmids encoding ESBL.

In order to test this hypothesis a further passage experiment was designed. The passage would follow the same design as the previous two passage experiments (Sections 7.2 page 134 & Section 7.3 page 140). Modifications to the methodology were as follows:

a) *E. coli* J62-2 (pCFF14) and *E. coli* J62-2 strain number 8/8 (pUK3007) were sub-cultured simultaneously.

b) each strain was successively sub-cultured into 5ml liquid media (IST broth) containing no antibiotic (control), 512mg/l ampicillin, or 8mg/l co-amoxiclav 20 times (20 days).

c) a loopful of each overnight culture was used to inoculate fresh media containing the required concentration of antibiotic.

d) ten colonies were purified from sub-cultures on days 5, 10, 15 and 20 and checked for the *lac his* Rif^R phenotype.

e) The MICs of ampicillin, co-amoxiclav and ceftazidime were determined for 96 of the 240 purified strains, together with selected control strains (a summary of the antibiotic susceptibilities is given in Table 3.21 and in Figures 3.27, 3.28 & 3.29 on pages 159-162).

Seven strains were selected from the 96 strains, which had had their antibiotic susceptibilities determined, for determination of the β-lactamase isoelectric points by analytical IEF (Figure 3.30 page 163). Each of the seven strains exhibited a single strong β-lactamase band with satellite bands of various pI values. Five of the seven strains exhibited a strong enzyme band of pI 5.5 (identical to the pI of TEM-5). Two of the seven strains (strain numbers 4.43 & 5.31) exhibited a strong enzyme band of pI 5.4 (identical to the pI of TEM-1). Some of the satellite bands

of the strains studied corresponded to the isoelectric points of TEM-10 (pI 5.57), TEM-5 (pI 5.5) and TEM-1 (pI 5.4). No enzyme bands of equivalent pI to that of TEM-12 were observed.

ANALYSIS OF SELECTED *E. COLI* J62-2 STRAINS

The plasmid DNA of several selected strains were isolated and visualised by agarose gel electrophoresis, and compared to plasmid DNA from the parent strains (*E. coli* J62-2 (pCFF14) and *E. coli* J62-2 (pUK3007)). Each of the strains examined were shown to contain a single plasmid. The plasmid DNA of various strains were then compared to pCFF14 and pUK3007 by restriction analysis (Figure 3.31 page 164) with the restriction endonucleases *Bgl* II which has no recognition site within the *bla*T ORF. Digestion with the restriction enzyme *Bgl* II provided maximum separation of the duplicate *bla*T genes of pUK3007 (see Figure 3.25 page 152). Plasmid DNA from the selected strains were found to have similar restriction patterns to pCFF14 and pUK3007.

Southern blotting and hybridisation (Figure 3.32 page 165) of the restricted DNA fragments using a 900-bp biotin-labelled probe, generated by PCR from *bla*T-1_{pAT153}, revealed that each mutant plasmid encoded either one or two TEM β-lactamase genes. One of the mutant TEM-1 producing strains (Strain number 4.43) encoded only a single TEM β-lactamase gene; whereas strain number 5.31 encoded two *bla*T genes. The *bla*T probe hybridised with additional bands that were present, as a result of incomplete restriction of the plasmid DNA.

A summary of the origin, antimicrobial susceptibilities, isolectric points of enzymes and results of the probing of the Southern transfer, for each of the strains studied in greater detail is given Table 3.22 (page 166).

Antibiotic Conc.‡	Inoculation Plasmid†	No. of strains	MIC (mg/l)§ AMP	AMX/CLAV	CAZ
0mg/l	pCFF14	6	<256	8	0.25
		4	<256	8	8
		5	<256	8	16
		1	512	8	16
	pUK3007	8	<256	8	0.25
		1	<256	8	0.5
		1	<256	8	16
		1	<256	8	64
		2	512	8	64
		1	1024	16	>64
		1	2048	8	64
		1	2048	64	>64
512mg/l ampicillin	pCFF14	3	2048	8	64
		1	2048	8	>64
		1	2048	16	64
		1	4096	8	>64
		1	4096	16	64
		9	4096	16	>64
	pUK3007	4	2048	8	>64
		4	4096	16	64
		8	4096	16	>64
8mg/l co-amoxiclav	pCFF14	2	<256	8	0.25
		4	2048	8	64
		4	2048	16	>64
		4	2048	32	>64
		2	4096	32	>64
	pUK3007	3	<256	8	0.25
		3	2048	8	>64
		5	2048	16	64
		1	2048	16	>64
		2	2048	32	64
		1	4096	32	64
		1	4096	32	>64
Controls:					
Strain no. 8/8 (pUK3007)			2048	8	>64
$TEM\text{-}1_{pAT153}$			<256	4	0.125
$TEM\text{-}5_{pCFF14}$			512	8	16

Table 3.21: Changes in antibiotic susceptibilities observed in the ampicillin/co-amoxiclav passage experiment.

‡ Antibiotic, with concentration used in passage experiment. † Plasmid harboured in the initial inoculating strain of *E. coli* J62-2. § Inoculum of 10^4 cfu/spot for each test organism. Abbreviations: AMP, ampicillin; AMX/CLAV, amoxycillin/clavulanic acid (ratio 2:1); CAZ, ceftazidime.

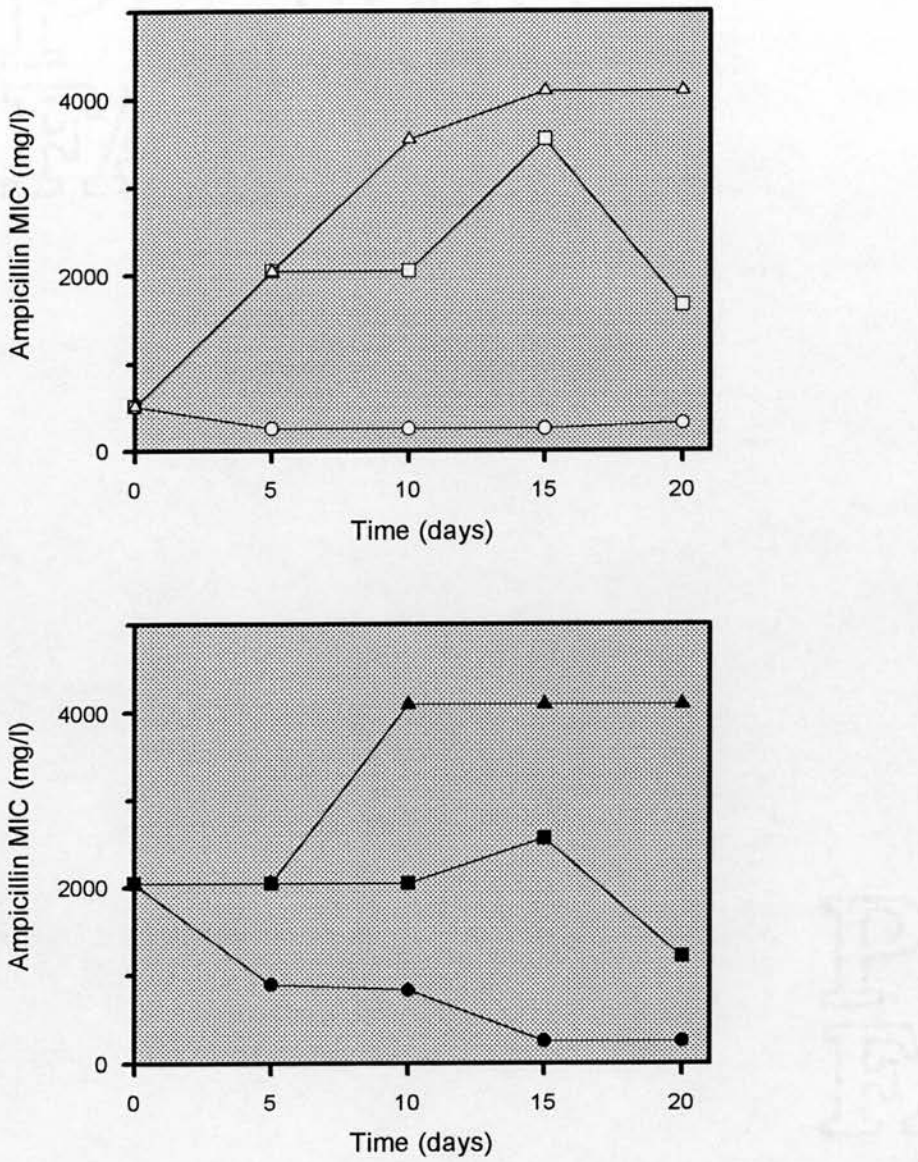

Figure 3.27: Changes in ampicillin MIC observed in the ampicillin/co-amoxiclav passage experiment.

The mean MIC (mg/l) of ampicillin for the purified colonies isolated, plotted on a daily basis, from the ampicillin/co-amoxiclav passage experiment. Time expressed in days from first inoculation.

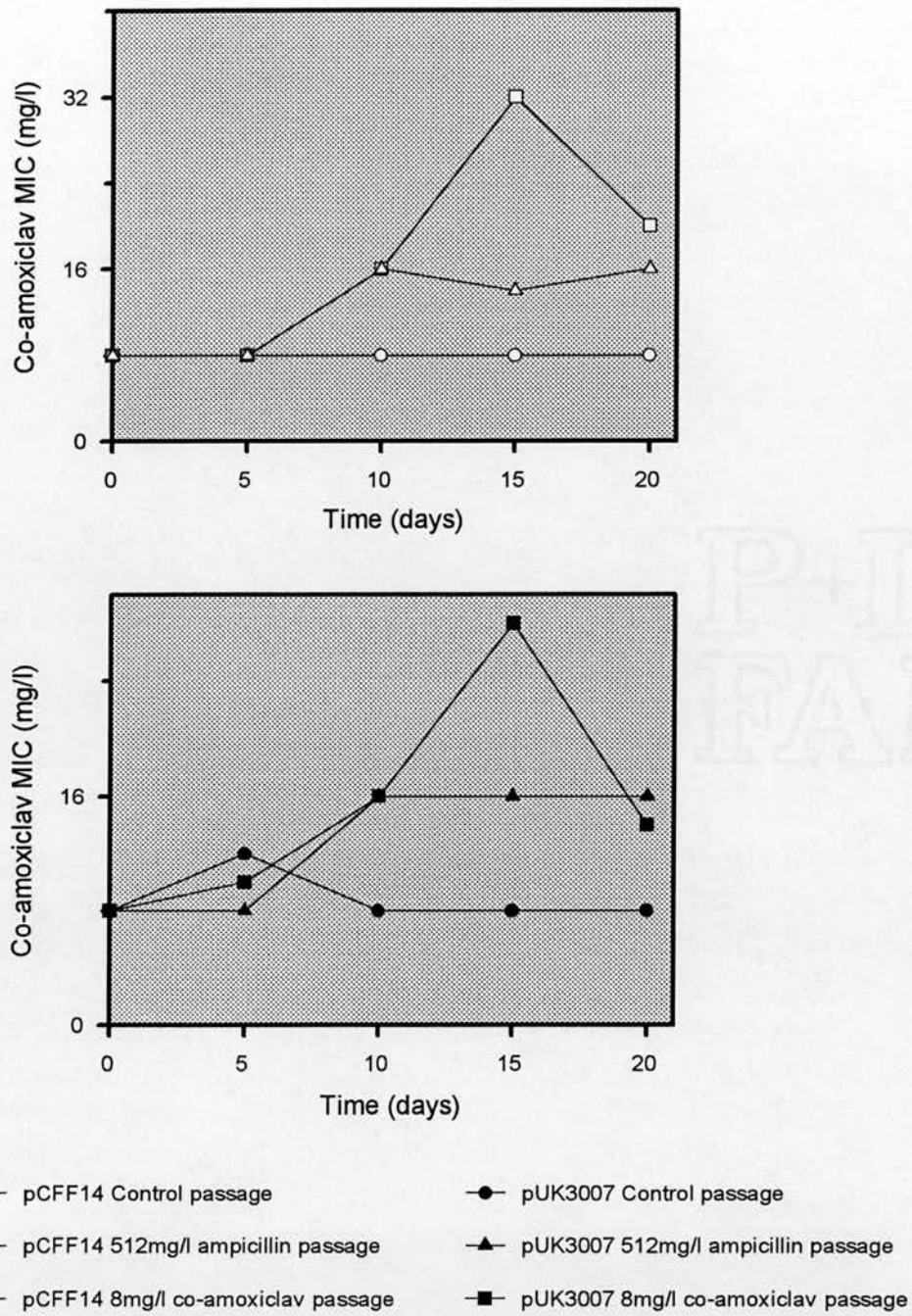

Figure 3.28: Changes in co-amoxiclav MIC observed in the ampicillin/co-amoxiclav passage experiment.

The mean MIC (mg/l) of co-amoxiclav for the purified colonies isolated, plotted on a daily basis, from the ampicillin/co-amoxiclav passage experiment. Time expressed in days from first inoculation.

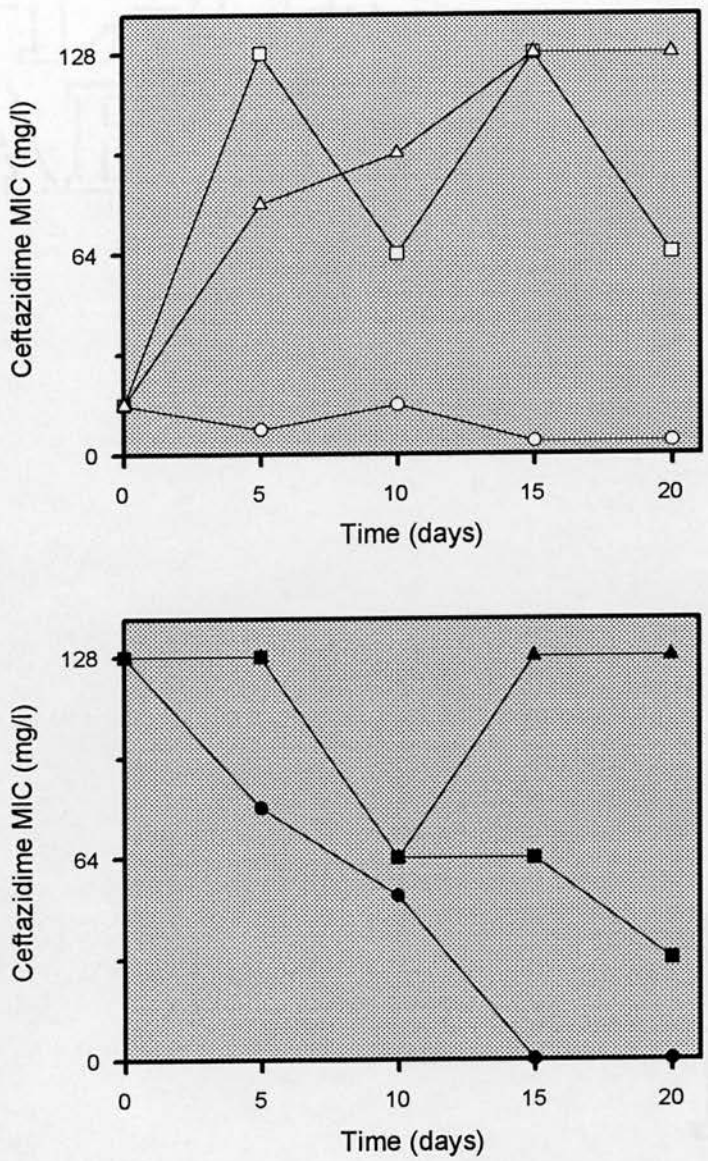

Figure 3.29: Changes in ceftazidime MIC observed in the ampicillin/co-amoxiclav passage experiment.

The mean MIC (mg/l) of ceftazidime for the purified colonies isolated, plotted on a daily basis, from the ampicillin/co-amoxiclav passage experiment. Time expressed in days from first inoculation.

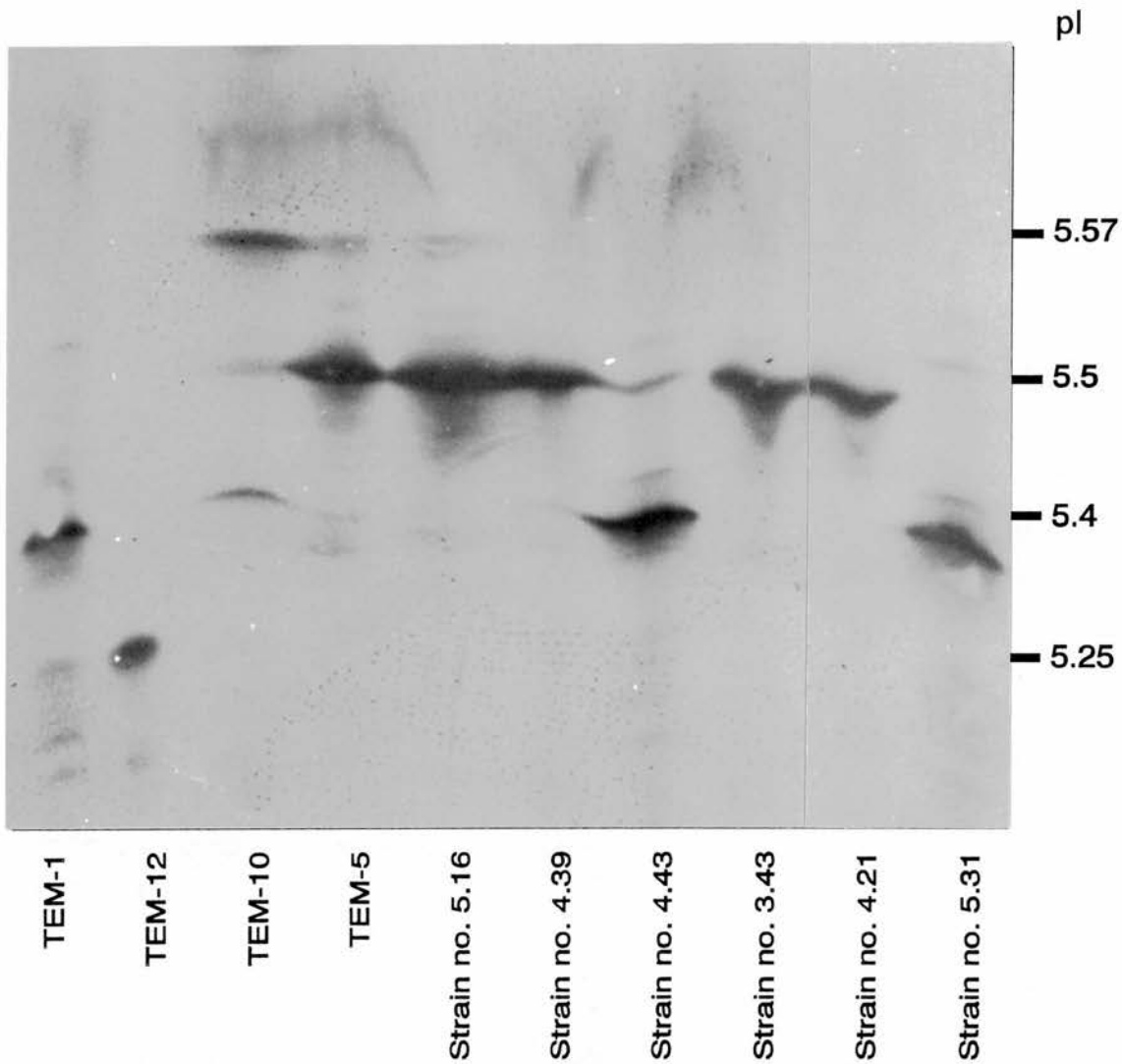

Figure 3.30: IEF gel showing the TEM-*type* β-lactamases from ampicillin/co-amoxiclav passage experiment.

IEF gel contained carrier ampholytes pH range 4-6. Crude extracts of test organisms were prepared and isoelectric points of the enzymes produced by each strain determined, and compared with preparations of enzymes of known pI. Standard β-lactamases shown include TEM-1 (pI 5.4), TEM-5 (pI 5.5), TEM-10 (pI 5.57), and TEM-12 (pI 5.25). The TEM-10 preparation also exhibits a TEM-1 band.

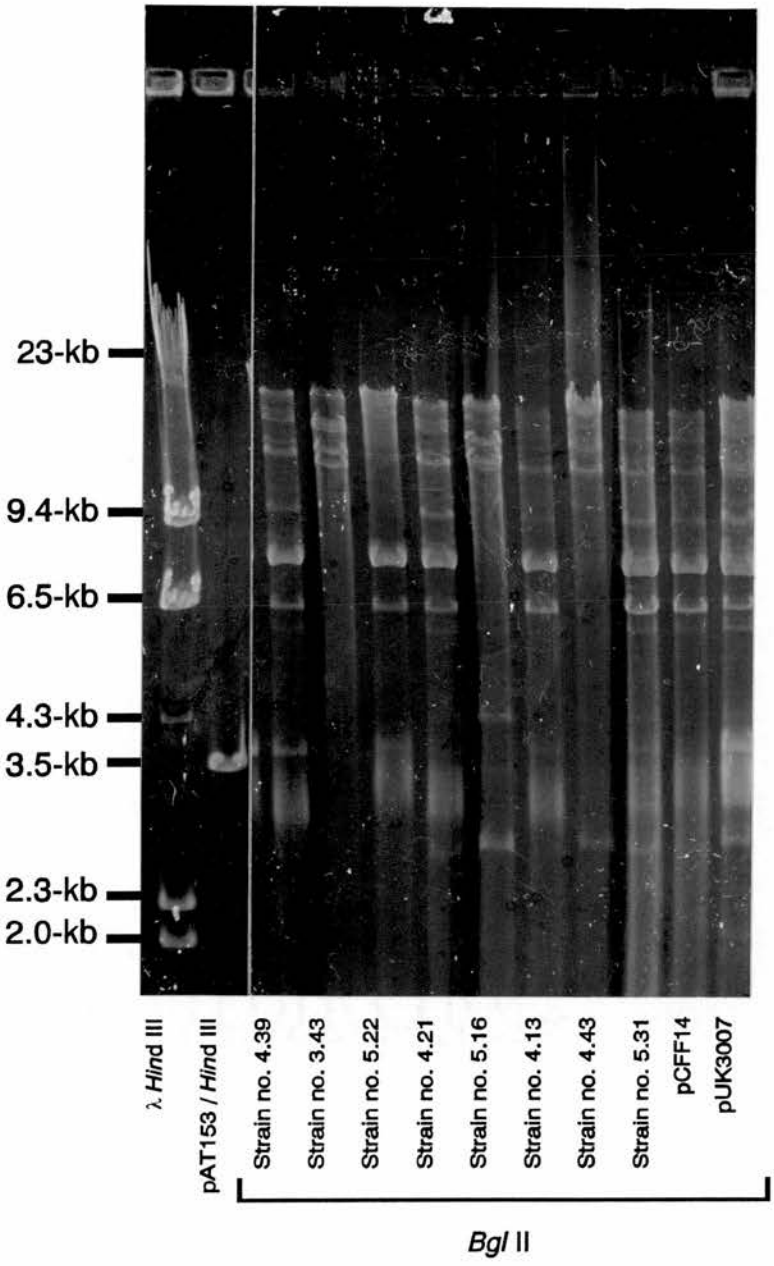

Figure 3.31: Restriction analysis comparing strains from the ampicillin/ co-amoxiclav passage experiment

Plasmid DNA from a number of strains from the ampicillin/co-amoxiclav passage experiment were digested with the *Bgl* II restriction endonuclease, and compared with the parent plasmids (pCFF14 and pUK3007). λ DNA digested with *Hind* III was used as an indicator of molecular weight. pAT153 was digested with *Hind* III as a control. Migration of the various DNA species was distorted as a result of variations in the electric field.

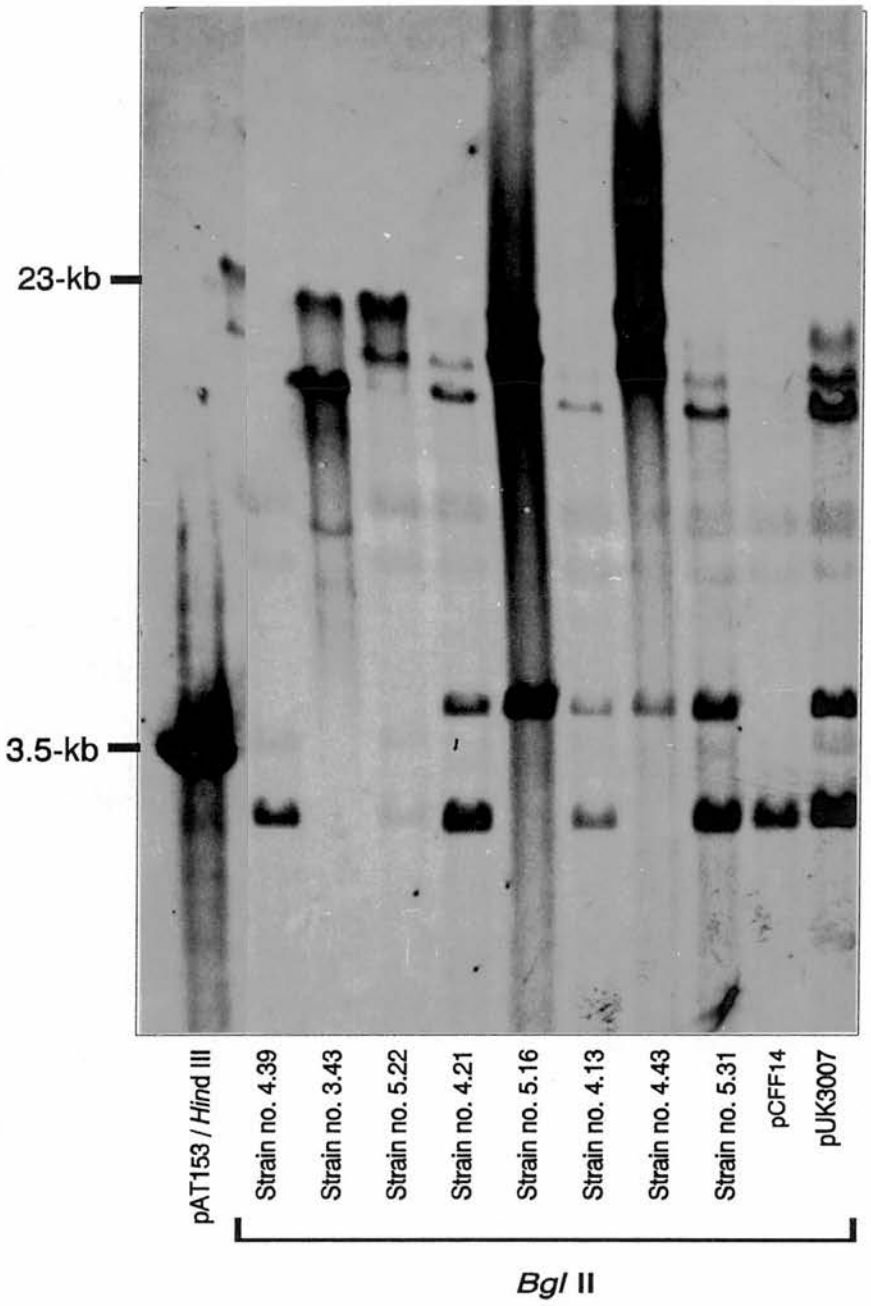

Figure 3.32: Southern blot of restriction analysis comparing strains from the ampicillin/ co-amoxiclav passage experiment

Southern transfer of restricted DNA (see Figure 3.31 page 164) was probed with a PCR-generated biotin labelled 900-bp *bla*T-1_{pAT153} fragment to reveal the DNA fragments encoding TEM β-lactamase genes. Linearised pAT153 was used as a positive control.

KINETIC ANALYSIS OF MUTANT β-LACTAMASES

The kinetic profiles of the mutant strains' β-lactamases were studied with the Perkin-Elmer UV/Vis 554 Spectrophotometer. The K_m and V_{max} values of the partially purified enzyme preparations were determined for the substrates penicillin G, nitrocefin, ceftazidime and cefotaxime; and the I_{50} value for clavulanic acid in the presence of penicillin G were determined. The results of the kinetic studies are given in Table 3.23 (page 167). The enzyme kinetics of the two β-lactamases from this third passage experiment, taking into account the isoelectric points of the respective enzymes, suggested that the β-lactamases were TEM-5 (pI 5.5) and TEM-1 (pI 5.4).

Strain no.	Passage details			Enzyme pI	No. of *bla*T genes	MIC (mg/l)‡		
	Source plasmid	Antibiotic	Day of isolation			AMP	AMX/ CLAV	CAZ
3.43	pCFF14	Ampicillin	15	5.5	NK	4096	16	>64
4.13	pCFF14	Co-amoxiclav	15	5.5	2	2048	32	>64
4.21	pUK3007	Co-amoxiclav	15	5.5	2	2048	32	64
4.39	pCFF14	Ampicillin	20	5.5	1	512	8	16
4.43	pUK3007	Co-amoxiclav	20	5.4	1	<256	8	0.25
5.16	pUK3007	Ampicillin	20	5.5	1	4096	16	>64
5.22	pCFF14	Co-amoxiclav	20	5.5	1	4096	32	>64
5.31	pUK3007	Co-amoxiclav	20	5.4	2	<256	8	0.25

Table 3.22: Details of selected *Escherichia coli* J62-2 strains from the ampicillin/co-amoxiclav passage experiment

‡ Inoculum of 10^4 cfu/spot for each test organism. Abbreviations: AMP, ampicillin; AMX/CLAV, amoxycillin/clavulanic acid (ratio 2:1); CAZ, ceftazidime; Co-amoxiclav, amoxycillin/clavulanic acid (ratio 2:1); NK, not known.

		Enzyme	
		Unknown	Unknown
Isoelectric point(s)		5.4	5.5
Source strain:		4.21	5.31
K_m (μM)	PEN G	16	98
	NCFN	39	9
	CAZ	UM	88
	CTX	UM	19
Relative K_m§	PEN G	41	1089
	NCFN	100	100
	CAZ	-	978
	CTX	-	213
V_{max} (μmol min^{-1})*	PEN G	96	1.1
	NCFN	13	1.5
	CAZ	UM	0.7
	CTX	UM	0.6
Relative V_{max}§	PEN G	7.4	73
	NCFN	100	100
	CAZ	-	49
	CTX	-	39
V_{max}/K_m	PEN G	5.8	0.01
	NCFN	0.35	0.17
	CAZ	-	0.01
	CTX	-	0.03
Relative V_{max}/K_m§	PEN G	166	6.5
	NCFN	100	100
	CAZ	-	4.9
	CTX	-	18
I_{50} (μM)‡:	CLAV	2.0	1.1

Table 3.23: Enzyme kinetics of the β-lactamases from the ampicillin/co-amoxiclav passage experiment.

Abbreviations: CAZ, ceftazidime; CLAV, clavulanic acid; CTX, cefotaxime; NCFN, nitrocefin; PEN G, penicillin G; UM, unmeasurable. § All values relative to nitrocefin (100%). * V_{max} values given as μmoles per minute, per ml of enzyme preparation. ‡ Enzyme and inhibitor (clavulanic acid) incubated for 5 minutes at 37°C before the reaction was initiated by the addition of penicillin G to 1mM.

Chapter 4

DISCUSSION

1. PROLOGUE

In Chapter 1 I tried to define some of the questions that required answering when this project was started, together with the questions that have been raised by the progress of other researchers. The aims of this thesis were to ascertain how the SHV- and TEM-*derived* ESBL have evolved under the various selective pressures exerted upon them. Every nucleotide mutation, and subsequent amino acid substitution, within an SHV- or TEM-β-lactamase will affect the binding of the substrates to the enzyme to some degree. Many of the mutations designated 'silent' may be found to affect enzyme binding or stability in the future, as assay techniques become more accurate and precise.

Chapter 3 describes the results of the experiments I performed for the completion of this thesis. From an example of the development of clinical resistance, I went on to describe a novel representation of the inter-relationships of very closely related proteins. Once the inter-relationship of each β-lactamase to another has been defined from its primary structure, a strategy for the investigation of evolutionary and enzymatic inter-relationships could be devised. The study of the enzymatic inter-

relationships led to the formulation of a hypothesis on the relative transient nature of many of the ESBL investigated.

I present a discussion, in this section, of the experimental work conducted for the completion of the aims of this thesis, together with the accumulation of the findings of other researchers' investigations into a hypothesis, which I have presented [245, 246], on the evolution of the SHV- and TEM-*derived* β-lactamases.

2. THE IDENTIFICATION OF β-LACTAMASES FROM CLINICAL ISOLATES

Ten clinical isolates of ceftazidime resistant *Kleb. pneumoniae* (KR1 to KR10) from the Royal Free Hospital (London) were characterised [233] in Autumn 1990. Eight of the ten strains originated from a single patient, Case 1, one strain originated from Case 2, and one from Case 3.

2.1. IDENTIFICATION OF THE β-LACTAMASES FROM CLINICAL ISOLATES KR1 TO KR9

The original isolate of *Kleb. pneumoniae* (KR1) from Case 1 was shown to express only a single β-lactamase with an isolectric point similar to that of the SHV-*type* or chromosomally encoded β-lactamases. Subsequent isolates (KR2→ KR8) from this patient expressed two β-lactamases: one of pI ~7.0, hence probably of chromosomal origin, and one of pI 5.4. The variation in expression of the pI ~7.0 enzyme, indicated by the variability of band intensity compared to the pI 5.4 after analytical IEF, suggested that this enzyme was also of chromosomal origin. Many Richmond & Sykes Class I β-lactamases [102] are produced only in 'basal' amounts [247]: the chromosomal β-lactamases of *Klebsiellae*, classified as Richmond & Sykes Class Ib enzymes, are constitutively produced [102]. The chromosomal β-lactamases of the various *Klebsiellia* spp. are not considered as clinically important as the chromosomal β-lactamases of other Enterobacteria [102, 247].

The acquisition of the pI 5.4 enzyme (strains KR2→KR8) coincided with the instigation of aztreonam therapy (Chapter 3 Figure 3.1 page 84), and the β-lactamase profile of each strain remained the same during further treatment, even

though a number of other β-lactam antimicrobial agents were used. The MICs of the β-lactam antibiotics provided by Dr MacDonald (Chapter 3 Table 3.2 page 87) show that for strain numbers KR2 to KR8, in comparison with strain KR1, an increase in all MICs were observed. The MICs of the β-lactam agents in combination with 2mg/l of clavulanic acid was also elevated. The identity of the pI 5.4 enzyme was probably TEM-1, because the relative rates of hydrolysis, shown in Table 3.3 (Chapter 3 page 90), show that strain numbers KR2 to KR8 exhibit mainly penicillinase activity. Strain number KR1, however, exhibits a much broader range of activity, hydrolysing both penicillins and cephalosporins.

The β-lactamases expressed by strain number KR9, isolated from Case 2, demonstrate the same characteristics as those enzymes expressed by strain numbers KR2 to KR8. The higher degree of cephalosporinase activity can probably be attributed to greater expression of the pI ~7.0 enzyme.

Comparison of the MICs of the β-lactam agents with and without the addition of 2mg/l clavulanic acid (Table 3.2) gives an indication of the contribution of the β-lactamases to the resistant phenotype of each individual strain. If the MIC of a particular β-lactam is not reduced in the presence of clavulanic acid, it is an indication that the β-lactamase expressed by the strain is not contributing to an increase of the MIC value of that drug, especially if the MIC values of other β-lactam agents are reduced when clavulanic acid is added to the test medium. The MICs of temocillin (a carboxypenicillin) and of cefoxitin (a cephamycin) are not reduced in the presence of 2mg/l clavulanic acid; however, the MICs of the 3GCs are reduced by the addition of a β-lactamase inhibitor for most of the strains. MICs for strain number KR1, which only expresses the pI ~7.0 enzyme, indicate that this enzyme binds and hydrolyses the 3GCs but not imipenem, temocillin or cefoxitin.

The data obtained from analytical IEF, enzymatic profiles, and the MICs of the various β-lactam agents suggest that the ceftazidime resistance phenotype observed is the result of OMP mutations in combination with the expression of a chromosomally encoded β-lactamase and the TEM-1 β-lactamase. The changes in MICs of the antibiotics tested can probably be attributed to changes in the porins of the host *Kleb. pneumoniae* strains. The change in the structure of the porins can affect the penetration of many of the β-lactam antimicrobial agents through the

bacterial cell envelope [40, 248-250]; differences in the charge of OMPs affect the mobility of ionic compounds through the outer membrane, acting as selective filters [251]. It is unfortunate that, because of the constraints of time, the OMPs of these strains were not studied.

2.2. IDENTIFICATION OF THE β-LACTAMASES FROM CLINICAL ISOLATE KR10

Kleb. pneumoniae strain number KR10 expressed three β-lactamases (isolectric points of 5.4, 5.57, and ~7.0). The pI 5.4 enzyme was probably TEM-1 and the pI ~7.0 enzyme was probably of chromosomal origin, as for strain numbers KR2 to KR9. The pI 5.57 enzyme was of particular interest. The presence of the TEM-*type* pI 5.57 enzyme greatly increases the MICs of the 3GCs, hence it was suspected that the pI 5.57 enzyme was an example of an ESBL.

The isoelectric point of the enzyme indicated that the β-lactamase was a TEM-*type* enzyme, which characteristically focus between pH 5 and pH 6. The pH at which the enzyme focused was the same as that of TEM-E3 and TEM-10, but was slightly more basic than TEM-5. The pI 5.57 β-lactamase was shown to be transferable after conjugation of the original *Kleb. pneumonaie* strain with *E. coli* J62-2; however, the pI ~7.0 enzyme was not observed to transfer, supporting the hypothesis that this β-lactamase was probably of chromosomal origin.

The comparison of the relative rates of hydrolysis of the original strain, and the transconjugants, with those of TEM-E3 and TEM-10, revealed similarities in profile with these β-lactamases. TEM-5 exhibits a much broader cephalosporinase activity than TEM-E3 and TEM-10. From this data the identity of the unknown pI 5.57 enzyme was probably TEM-10/TEM-E3. Precise identification could only be given if the amino acid sequence of the β-lactamase was determined.

2.3. THE DEVELOPMENT OF RESISTANCE IN CLINICAL ISOLATES

Case 1 represents the *in vivo* selection of resistance as a direct result of the antibiotic therapy administered. Although resistance was not a consequence of ESBL-encoding plasmid transfer, TEM-1 was shown to be acquired during

therapy. The failure to treat, successfully, the pyrexia that this patient was suffering from, was remedied when imipenem therapy was instigated. Imipenem was not hydrolysed by either of the β-lactamases present in the strains isolated from Case 1 (on the basis of the changes in MICs in the absence and presence of clavulanic acid). The imipenem was, therefore, only subject to a reduction in penetration into the bacterial cell, as the result of porin changes. The OMP mutations alone were not sufficient to cause treatment failure.

Case 2 did not require antibiotic therapy, but *Kleb. pneumoniae* were isolated from the urine during her stay at the RFH. This patient illustrated the possible problems of inter-hospital transfer, especially international transfer of patients that may be carrying resistant organisms, that may easily be disseminated to other patients if hospital staff are not vigilant. It is fortunate that, in this case, the clinicians involved were careful and treated Case 2 in isolation.

Kleb. pneumoniae strain number KR10 was isolated from Case 3 on the day of admission. The ESBL expressed by strain number KR10 was probably selected by therapy the patient had received before admission to the RFH, or as the result of cross-infection of a resistant organism. The origin of this particular ESBL is not clear.

All three patients could have disseminated resistant organisms within the hospital, fortunately no resistant organisms, from any of these three sources of infection, were transferred to other patients.

3. ANALYTICAL ISOELECTRIC FOCUSING

TEM-10 has been reported to have various isolectric points (pI 5.55, 5.57 and 5.6) [152, 188, 192, 252] and TEM-5 has been reported to have a pI of between pH 5.5 and 5.6 [152, 152, 184, 188, 193, 252, 253]. Comparison of TEM-E3 with TEM-10 indicates that these two β-lactamases focus at exactly the same isoelectric point, confirming the observations on the similarity of TEM-E3 to TEM-10 [192]. The enzymes TEM-10 and TEM-5 are, however, not found to focus at the same pH (see Chapter 3 Figure 3.23 page 149), as TEM-10 focuses at a slightly lower pH than enzymes given a pI of 5.6.

The ability to accurately identify the pI of an enzyme to within one hundredth of a pH unit is dubious: a difference between pH 5.5 and pH 5.6 equates to a difference of about 0.6μM in hydrogen ion concentration (for simple acids). The isolectric points of enzymes can be ascertained by focusing alongside β-lactamases with similar pI values and estimating the pI, if the unknown enzyme does not focus exactly at the same point. The pH gradient can be determined, after focusing, by placing a surface pH electrode on the polyacrylamide gel, at several positions, but this only provides an estimate of pH. The only sure method of determining whether a particular enzyme has exactly the same pI as another β-lactamase is to focus both enzymes alongside each other. If the enzymes do not focus at the same pH an estimate of the pI value of the unknown β-lactamase is then made. Proteins that do not co-focus will not be the same, they must exhibit some variation in primary structure when compared to each other. A number of the pI values of the TEM- and SHV-*type* β-lactamases have been determined from the measurement of the pH gradient with a surface pH electrode [105, 163].

The apparatus used for isoelectric focusing has a bearing on the resolution of the technique. The LKB 2217 Ultraphor Electrofocusing unit (Pharmacia) is used by many workers with the LKB Ampholine PAGplates (ready-made IEF polyacrylamide gels; Pharmacia). The LKB Ampholine PAGplates are only manufactured with fixed, linear, pH ranges. Non-linear and customised pH ranges, that provide better separation and focusing of β-lactamases with very similar pI values, have to be prepared according to the method described in Chapter 2 [105, 203]. By combining carrier ampholytes of different pH ranges a non-linear gradient is formed during focusing: mixing Ampholines of pH range 3.5-10 with Ampholines of pH range 4-6 (ratio 1:1) enlarges the resolution in the pH range 4-6 and compresses the gradient at a pH less than 4, and at a pH greater than 6. Some β-lactamases may appear to co-focus at the same pH when separated on a linear pH gradient, however, they may not co-focus on a non-linear gradient, showing that the pI values of the respective enzymes are actually slightly different. A disadvantage of the non-linear gradient is that external measurement of pH gradient (with a pH electrode) would be difficult.

The isolectric point of TEM-5 has, therefore, been taken as being pI 5.5, and TEM-E3 and TEM-10 are assumed to focus at pI 5.57 throughout this thesis.

4. THE CLASSIFICATION OF THE SHV- & TEM-*DERIVED* EXTENDED SPECTRUM β-LACTAMASES

It is current practice to classify β-lactamases by their nucleotide sequence and for most enzymes this works well; however, all the extended-spectrum β-lactamases fall into Ambler class A [109] which encompasses enzymes whose genes share as little as 35% identity [120] but retain the same overall tertiary structure. The lack of correlation between primary structure and enzyme function is epitomised with the prototype enzymes TEM-1 and SHV-1: these enzymes are impossible to distinguish enzymologically. The TEM-5 β-lactamase gene, *bla*T-5, encodes only three amino acid changes from that of TEM-1 [160]. The expanded substrate profile of the TEM-5 β-lactamase, compared to TEM-1, confers a resistant phenotype of immense clinical importance [254]. A classification scheme based entirely on the structure is, therefore, unhelpful.

4.1. THE EXTENSION OF THE PAYNE & AMYES CLASSIFICATION

The Payne & Amyes categorisation scheme [152] is based on the biochemical properties and the resistances that the enzymes confer show that the mutations produce three major changes in function (described in Chapter 1). ESBL in Group 1, which give low level resistance to cefotaxime or ceftazidime, possess amino acid substitutions that increase the ability of the enzyme to hydrolyse ceftazidime faster than cefotaxime. Groups 2 and 3 will produce high levels of resistance to ceftazidime and/or cefotaxime. ESBL in Group 2 hydrolyse ceftazidime to a greater extent than cefotaxime, whereas Group 3 enzymes hydrolyse cefotaxime better than ceftazidime. Professor Amyes and I have suggested an additional group (group zero) [255]; the enzymes of which are virtually indistinguishable from the prototype enzymes TEM-1, TEM-2 and SHV-1 on the basis of resistance profile, for example TEM-13 [157], TEM-17 [157, 241], TEM-18 [157] and SHV-8 [235]. Often the *in vitro* ability to hydrolyse cefotaxime is greater than that required to hydrolyse ceftazidime, but the fast penetration of cefotaxime ensures that this hydrolysis is insufficient to overcome the drug's antibacterial activity. These changes seem to work in concert with previous mutations.

A universally recognised indicator of bacterial sensitivity to antibiotics is the minimum inhibitory concentration, the lowest concentration required to inhibit bacterial multiplication. This can be used to compare each clinically observed mutation inserted into the maps of the ESBL inter-relationships (Chapter 3 Figures 3.5 & 3.6, pages 95 & 98 respectively) with the biochemical properties of the enzyme and the resistance profile that it confers [234], allowing a structure-function relationship to be devised and suggestions for evolution to be proposed.

4.2. THE SHV-*DERIVED* β-LACTAMASES

The relationships of the SHV-*derived* enzymes are well defined and are shown in Figure 4.1. Sequence data show that all clinical SHV-*derived* ESBL have the same change of amino acid at position 238 and thus must have all derived from SHV-2. This first mutation from SHV-1, changing glycine to serine, gives a Payne & Amyes Group 3 enzyme with significant hydrolysis of both cefotaxime and ceftazidime. This produces a very large increase in the MIC of cefotaxime but only a moderate increase in the MIC of ceftazidime [153, 155] (Table 4.1).

Enzyme	MIC (mg/l)§		
	Cephalothin	Ceftazidime	Cefotaxime
SHV-1_{p453}	16	0.2	0.01
SHV-2_{pBP60}	256	2	4
SHV-3_{pUD18}	128	2	2
SHV-4_{pUD21}	256	64	4
SHV-5_{pAFF2}	256	32	4

Table 4.1: Resistance profiles of the SHV-*derived* β-lactamases

Data taken from Philippon, Labia & Jacoby (1989) [153] and presented as representative MIC values. § Determined by agar dilution of *E. coli* J53-2.

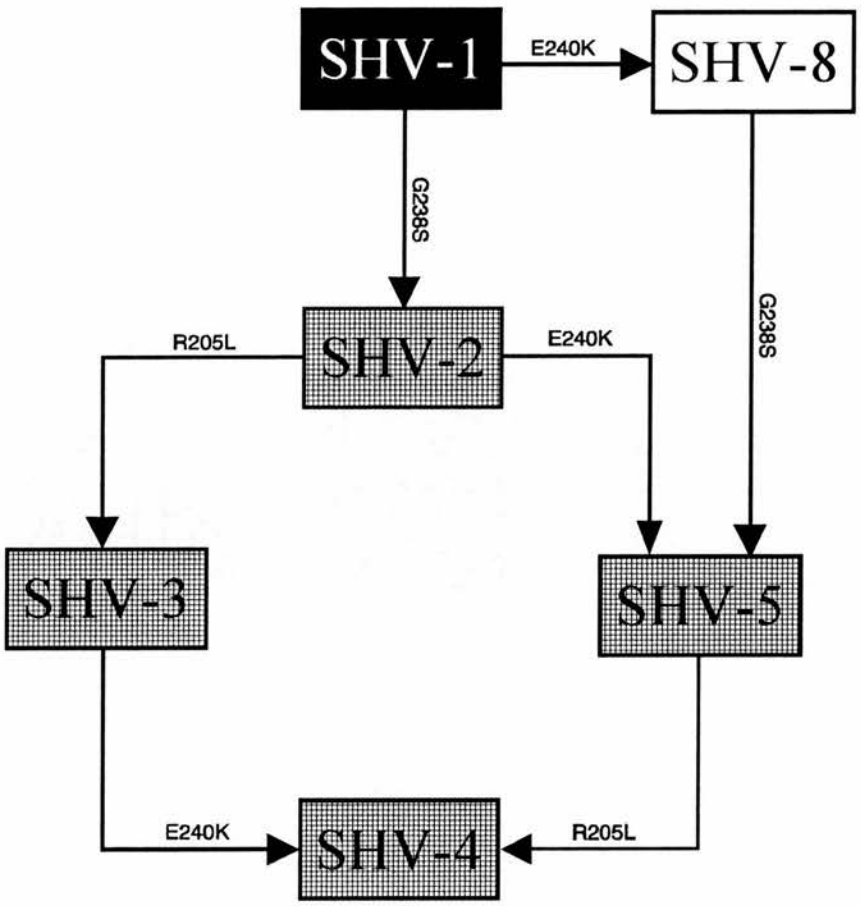

Figure 4.1: The inter-relationships of the SHV-*derived* β-lactamases

Each arrow represents a single amino acid change. Boxes, each representing a β-lactamase, are shaded according to the enzyme's substrate profile (see the legend below), based upon the Payne & Amyes classification [152]. One letter amino acid notation according to the IUPAC-IUB Commission on Nomenclature (1969) [237]. Amino acid numbering according to the recommendations of Ambler *et al.* (1991) [158].

Parental enzyme with no ESBL activity

Group 0: Very low or non-existent ESBL activity

Group 3: High ESBL activity (ceftazidime < cefotaxime)

SHV-4 was derived from SHV-2 by substitution of two amino acids, either through SHV-3 or SHV-5. Study of the MICs of cefotaxime and ceftazidime, for bacteria harbouring these intermediate SHV-*derived* β-lactamases [153], shows that the change of the amino acid at position 205 from arginine (SHV-2) to leucine (SHV-3) does not increase cefotaxime or ceftazidime resistance. On the other hand, a change in the amino acid at position 240 from glutamic acid (SHV-2) to lysine (SHV-5) has little effect on the MIC of cefotaxime, but considerably increases the MIC of ceftazidime. Further challenge of an SHV-2 producing strain with a 3GC, particularly ceftazidime, is likely to select the SHV-5 mutation rather than the SHV-3, which gives no apparent further selective advantage. Furthermore, when the SHV-5 enzyme has an additional substitution of arginine by leucine at position 205, these changes work in concert and there is a further increase of both cefotaxime and ceftazidime resistances. Huletsky *et al.* [235] corroborate the evidence presented by Sowek *et al.* [241], who found that the E240K change in the TEM-*derived* β-lactamases raised the MIC of ceftazidime slightly, but had no effect on the MIC of cefotaxime. Huletsky *et al.* [235] found that the glutamic acid-240→lysine resulted in a small increase in the MIC of ceftazidime, but a very nominal rise in the MIC of cefotaxime.

This analysis suggests that a single change from glycine to serine at position 238 vastly increases cefotaxime resistance, with a more limited rise in the MIC of ceftazidime. An isolated change at position 205 does not improve the resistance profile of an extended-spectrum SHV enzyme; however, the same substitution at position 205, in addition to a change at 240, has a co-operative effect on cefotaxime and ceftazidime resistance. A co-operative effect also exists between amino acid 238 and residue 240.

For a mutation encoding a Glu-240→Lys change to be evolutionary viable, the Gly-238→Ser change must have already occurred. Some co-operative spatial effect between the two amino acids must therefore occur. The same appears to be true of the residue at position 205. The Arg-205→Leu change does confer a slight, if small, increase in 3GC resistance when combined only with the glycine to serine mutation at residue number 238 (SHV-3). I suspect that a nucleotide mutation in the SHV-1 gene that encoded leucine-205, instead of arginine, would exhibit no increase in resistance to the 3GCs.

Another deduction from this data is that the glycine-238→serine substitution is most likely to be selected by an antibiotic like cefotaxime, whereas the remaining two mutations are more likely to be selected by ceftazidime treatment, provided that the glutamic acid-240→lysine change occurs before the arginine-205→leucine substitution. A probable sequence of changes under clinical selection is, therefore, likely to have been SHV-1→SHV-2→SHV-5→SHV-4.

4.3. THE TEM-*DERIVED* β-LACTAMASES

The mutation relationships are well defined amongst the TEM enzymes and are shown in Figure 4.2 (page 179), although they are more complex. It can probably be assumed that the mutation between TEM-1 and TEM-2 occurred before later enzymes emerged, and that the glutamine-39→lysine change does not occur with the ESBL. Two clearly separate TEM-*derived* enzyme groups, therefore, exist in the TEM-*derived* enzymes: TEM-1-*derived* and TEM-2-*derived*.

A three-dimensional structure of the TEM-1 β-lactamase has been published [126, 127]. Analysis of the tertiary structure (Figure 4.3 page 180) of the β-lactamases place the sites of amino acid variation adjacent to the proposed position of the respective β-lactam 6(7)-substituents when bound at the active site of the enzyme. The amino acid mutations alter the shape of the active-site, either directly or indirectly, and reduce hindrance of β-lactamase-cephalosporin binding. In some cases changes may promote binding through additional charge-charge interactions with the drug moieties. In other cases, binding of a substrate may be enhanced by the reduction in hydrogen bond lengths.

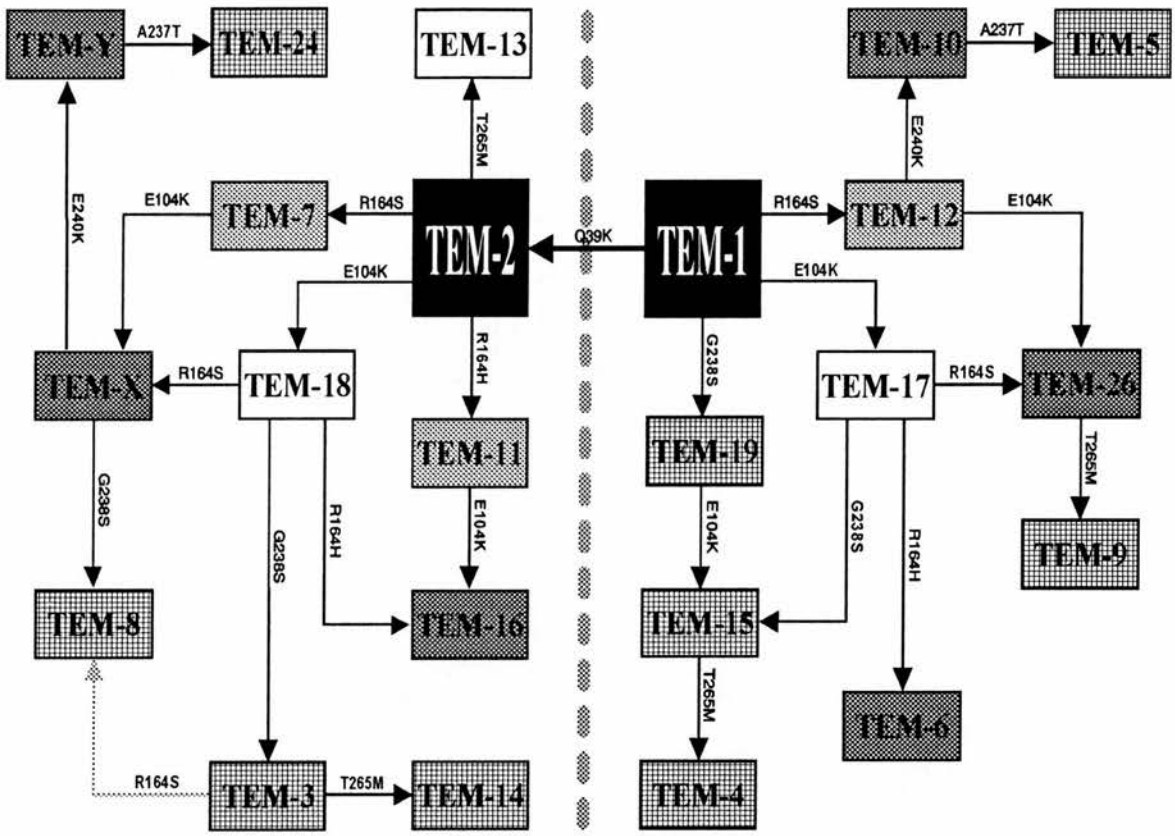

Figure 4.2: The inter-relationships of the TEM-*derived* β-lactamases

Each arrow represents a single amino acid change. Boxes, each representing a β-lactamase, are shaded according to the enzyme's substrate profile (see the legend below), based upon the Payne & Amyes classification [152]. The enzymes denoted TEM-X and TEM-Y have not been isolated clinically (see Chapter 3, Section 3.2 page 94 for details) One letter amino acid notation according to the IUPAC-IUB Commission on Nomenclature (1969) [237]. Amino acid numbering according to the recommendations of Ambler *et al.* (1991) [158].

- Parental enzyme with no ESBL activity
- Group 0: Very low or non-existent ESBL activity
- Group 1: Low ESBL activity (ceftazidime > cefotaxime)
- Group 2: High ESBL activity (ceftazidime > cefotaxime)
- Group 3: High ESBL activity (ceftazidime < cefotaxime)

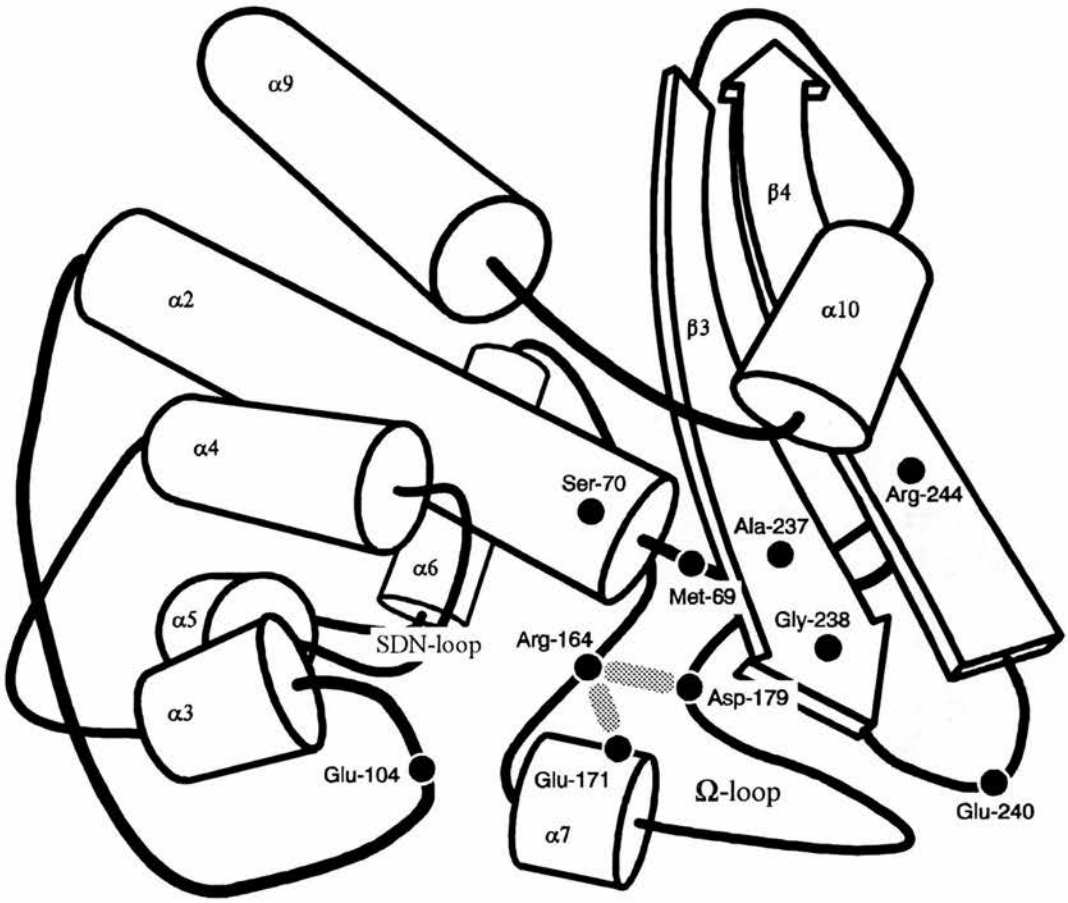

Figure 4.3: Diagram of the active-site cavity of the TEM-1 β-lactamase

Diagram showing the amino acids important in substrate binding which lie close to the TEM-1 active site, based upon the crystal structure described by Jelsh *et al.* (1992 & 1993) [126, 127]. Numbering of amino acid residues according to the recommendations of Ambler *et al.* (1991) [158]. Abbreviations: ▒▒▒ , salt-bridge ; Ala, alanine; Arg, arginine; Asp, aspartic acid; Glu, glutamic acid; Lys, lysine; Met, methionine; Ser, serine.

Considering the MIC values of various β-lactam antimicrobial agents for the TEM-1→TEM-5 and the TEM-1→TEM-9 β-lactamases from my results and published sources, some interesting observations can be made (Chapter 3: Table 3.13 page 127 [241] and Table 3.15 page 134). The first alteration from TEM-1 to the enzymes TEM-5 or TEM-9, is the alteration of arginine-164 to serine. The resultant β-lactamase, TEM-12, has a limited capability to hydrolyse either ceftazidime or cefotaxime and is classified as Du Bois, Payne & Amyes Group 1. The presence of this enzyme increases ceftazidime resistance but hardly affects susceptibility to cefotaxime. The same substitution can take place with the TEM-2 β-lactamase to give TEM-7, which has similar properties [186, 189]. The increased capability to hydrolyse oxyimino cephalosporins does not arise from the acquisition of serine at position 164, but from the loss of arginine. The Arg-164→His change in TEM-2 also gives an enzyme (TEM-11) with the same properties [256]. It is interesting that, in spite of the fact that the loss of arginine at position 164 appears so often in the TEM enzymes, this substitution is not found amongst the SHV group. The unusually short α-7 helix of the TEM-1 enzyme [126, 127], compared to other Class A enzymes, may explain the lack of substitution in the SHV group.

As with the SHV-4 enzyme (Figure 4.1 page 176) the development of TEM-9 can be accomplished by two distinct routes (Figure 4.2 page 179). The TEM-17 mutation, substituting glutamic acid-104 with lysine, is a potential alternative to TEM-12; however, this change does not significantly increase the MIC of cefotaxime and has no effect on the MIC of ceftazidime. Combination of the lysine-104 and serine-164 substitutions, as seen in TEM-26, results in a synergistic effect, increasing the MIC of ceftazidime out of proportion to the contribution of each separate mutation. The MIC of cefotaxime for TEM-26 is the same as that obtained for TEM-17. The TEM-26→TEM-9 transition (threonine-265→ methionine) increases the MIC of cefotaxime to a greater extent than the MIC of ceftazidime [179, 180]. The TEM-12 to TEM-10 change (glutamic acid-240→ lysine) greatly increases the ceftazidime resistance conferred. A further substitution of threonine for alanine at position 237 (TEM-5) further increases the laboratory resistance to cefotaxime but not to ceftazidime.

It is possible to plot and compare the development of TEM-5 and TEM-9; both enzymes have similar substrate profiles, the latter slightly more resistant to the

action of cefotaxime, but the MICs of ceftazidime remain equivalent for both. In their respective selections, the arginine-164→serine change appears to be the crucial first stage; it is a fairly common mutation, both with TEM-1 and TEM-2 and thus has a pivotal position in the development of over half the TEM-derived ESBL. The second step mutations, TEM-26 and TEM-10, do not differ in their abilities to confer resistance to cefotaxime; however, TEM-26 has a greater ability to confer resistance to ceftazidime. For both TEM-5 and TEM-9 the first two mutation events are likely to have been selected by ceftazidime, or a similar drug, and the final stage is likely to have been selected by cefotaxime, or similar antibiotic. Study of the threonine-265 and alanine-237 mutations show that the latter is more potent in conferring cefotaxime resistance than the former. This progression of mutations shows that the arginine to serine substitution at position 164 and the glutamic acid to lysine change at position 240 both increase ceftazidime resistance. The glutamic acid to lysine mutation at position 104 has a minimal effect on substrate profile, except in combination with the removal of arginine at position 164, when it promotes a further increase in ceftazidime resistance. The mutations at positions 237 and 265 both raise the MICs of ceftazidime and cefotaxime, although they have a more significant effect on the latter. The substitution of methionine for threonine at position 265, in the absence of other mutations (TEM-13), is effectively silent as it has no effect on MIC values [157].

5. SPATIAL EFFECTS OF POINT MUTATIONS

Figure 4.3 (page 180) shows the latest representation of the three-dimensional structure of the active-site of the TEM-1 β-lactamase, together with the location of each of the point mutations and other residues important in substrate binding. The arginine-164 residue is situated on the Ω-loop, part of which is made up by the short α-7 helix. A major difference between the structure of TEM-1 and other Ambler Class A β-lactamases appears to be the very short α-7 helix in the former [126, 127]. The arginine-164→serine change would disrupt the two salt bridges formed between the basic arginine-164 and the acidic residues aspartic acid-171 and aspartic acid-179 [127]. Other Ambler Class A β-lactamases with an arginine at position 164 only form a single salt-bridge [123]. The ionic bond across the Ω-loop retains the shape of the

back of the active-site, ideally sized for the earlier (and smaller) β-lactam antimicrobial agents. Prevention of salt bridge formation, by replacing the basic guanidine group of arginine with the neutral alcohol group of serine, results in the active-site cavity becoming less rigid. The movement of the α-7 helix, increasing the size of the oxyanion hole that forms the active-site, allows the tighter binding of 3GCs, especially ceftazidime.

Glutamic acid-104 is situated adjacent to the α-3 helix, and is partially obscured from full participation in substrate binding by the Ω-loop in the TEM-1 β-lactamase. A mutation to substitute lysine at this position does not contribute greatly to substrate binding, unless arginine is lost from position 164 and the salt bridges were not formed. The synergy that exists between the substitutions at positions 164 and 104 would be explained by this model. The increase in size of the active-site cavity, as a direct result of a change preventing salt bridge formation, will also expose residue 104 and allow full contribution to substrate binding.

The residues at positions 237, 238, and 240 are situated at the end of β-3 sheet (Figure 4.4 page 184). Mutation of these residues enlarges the active-site by the movement of the β-3 sheet away from the α-2 helix [235, 257]. Enlargement of this region of the β-lactamase active-site creates an enzyme with a higher affinity for the 7-oxyimino cephalosporins; substitutions at positions 237 and 238 favour cefotaxime resistance, whereas substitution at 240 favours ceftazidime resistance. The spatial contribution, if any, of changes observed at residue 265, situated at the end of the β-5 sheet, remain unclear.

5.1. Analysis of biochemical data

The conclusions drawn from the MIC values are confirmed by the biochemistry. V_{max}, K_m, k_{cat}, and V_{max}/K_m all support the MIC data. The turnover number, k_{cat}, reveals that an increase in the capacity of a specific enzyme to hydrolyse a 7-oxyimino cephalosporin is usually concurrent with a decrease in the turnover of the simpler penicillins, such as ampicillin and amoxycillin, in comparison with either TEM-1 or SHV-1 respectively. This observation suggests that the ESBL are less efficient at hydrolysing the earlier β-lactams than their ubiquitous prototypes.

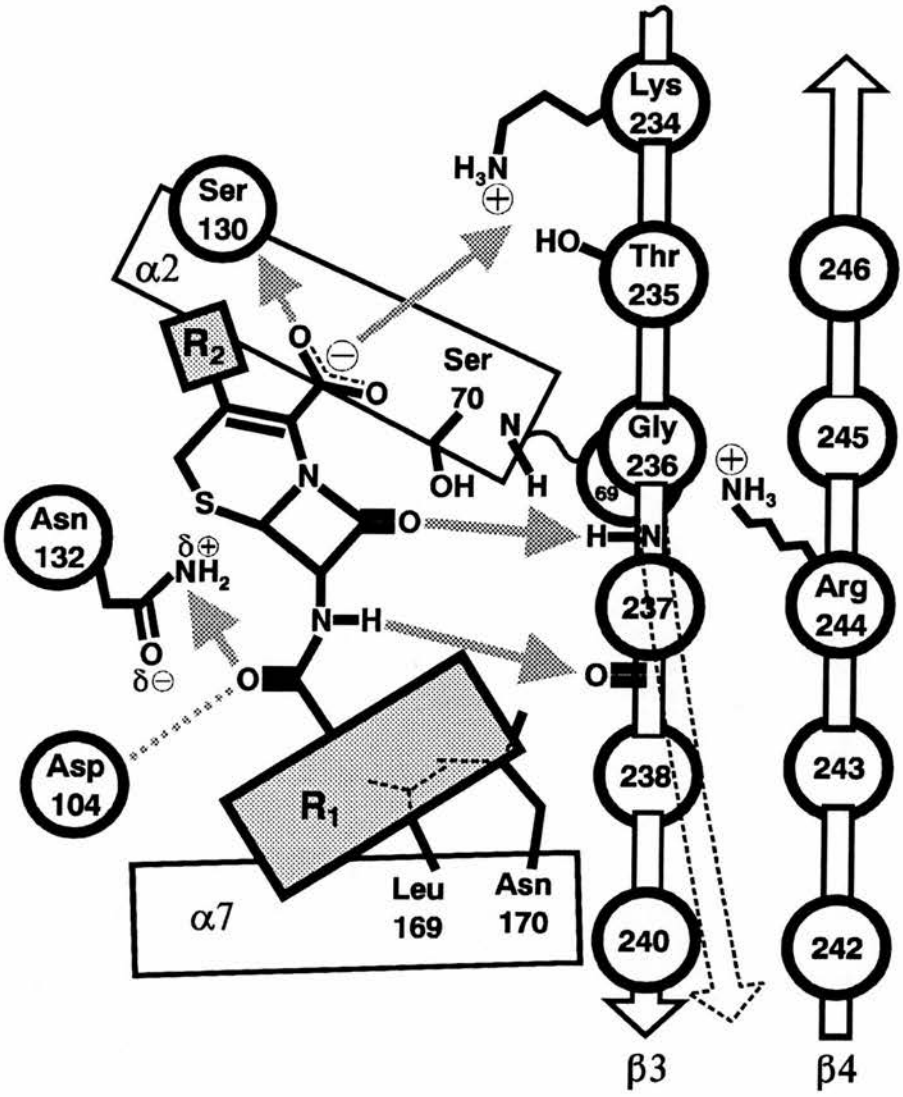

Figure 4.4: Schematic representation of β-lactam binding to the active-site of the SHV-*derived* β-lactamases

Diagram is based on the crystal structure of the *Bacillus licheniformis* 749/c β-lactamase. Electrostatic and hydrogen-bonding interactions, which can attract β-lactam drugs are indicated by the arrows (⇒). The dotted line (⋯⋯) to aspartic acid-104 indicates the hydrogen bond present in the *B. licheniformis* 749/c enzyme, and in the TEM-*derived* lysine-104 mutants, but not the SHV-*derived* β-lactamases. The mutation of residues number 237 and 240 moves the β-3 sheet away from the α-7 helix in the manner indicated. Amino acid numbering according to the recommendations of Ambler *et al.* (1991) [158]. Abbreviations: Arg, arginine; Asn, asparagine; Asp, aspartic acid; Gly, glycine; Lys, lysine; Ser, serine; Thr, threonine. Illustration after Knox (1993) [257].

5.2. THE EVOLUTION OF TEM-*DERIVED* β-LACTAMASES

The biochemical development of TEM-5 and TEM-9 from the prototype TEM-1 β-lactamase was examined above in terms of resistance. The data presented in Chapter 3 (from [241]) show that, while the turnover numbers for cefotaxime and ceftazidime increase, the k_{cat} for benzylpenicillin decreases. The greater the efficiency of 7-oxyimino cephalosporin hydrolysis, the slower the turnover of benzylpenicillin. The k_{cat} values for both ceftazidime and cefotaxime reflect the MIC results. Comparison of the benzylpenicillin turnover number of TEM-17 with that of TEM-12 shows that, although the latter enzyme hydrolyses the 3GCs with greater efficiency than the former, TEM-17 has a greater ability to degrade the early penicillins than TEM-12. The only time that such a situation would be an advantage would be if the host organism was challenged with a penicillin. Thus, if TEM-26 had been selected from TEM-12 by the clinical use of the 3GCs and the host was subsequently challenged with a penicillin, a mutation to TEM-17, rather than back to TEM-12, would theoretically have a selective advantage.

Simple selective pressure with ampicillin does not appear to induce such a mutation. The use of a β-lactamase inhibitor, for example clavulanic acid, would provide a much stronger selective pressure than amoxycillin or ampicillin on their own. Comparison of the I_{50}values of clavulanic acid for the enzymes suggested that the use of this inhibitor may induce back mutations, and experimental results seem to support this hypothesis (see below). The back mutation of TEM-26 to TEM-17 would allow the forward mutation to TEM-15, then subsequently TEM-4 (Figure 4.2 page 179), or to TEM-26 if re-challenged with a 7-oxyimino cephalosporin, whilst still providing a higher level of penicillin and clavulanic acid resistance for the host organism compared to some other first-step mutations (e.g. TEM-12 or SHV-5).

When considering the evolution of TEM-8 from those enzymes identified from clinical isolates, the progression would seem to be TEM-2→TEM-18→TEM-3→ TEM-8. However, TEM-18, a TEM-2 derived analogue of TEM-17, is a Group 0 enzyme (very low or non-existent 7-oxyimino cephalosporin resistance). TEM-7, equivalent to TEM-12, belongs to Du Bois, Payne & Amyes Group 2 (low level 3GC resistance), and as illustrated in the TEM-1→TEM-9 progression described

above provides a much greater selective advantage over TEM-18 as a first step mutation. Substitution of glutamic acid-104 for lysine in TEM-7 to create a TEM-2-*derived* clinical analogue of TEM-26, TEM-X, would appear to be the logical link to TEM-8. TEM-X has not been identified from a clinical isolate, but its existence is supported by the identification of TEM-24. TEM-8 is probably the progeny of TEM-X, rather than of that of TEM-3 via TEM-18.

6. SITE-DIRECTED MUTAGENESIS OF *bla*T-1$_{pAT153}$

When the first version of the map describing the inter-relationships of the SHV- and TEM-*derived* β-lactamases was introduced [234], only eight of the TEM-*derived* β-lactamases had been sequenced at either an amino acid or DNA level. Many of the possible intermediates had not even been identified as possible candidates on the basis of phenotype, but it was only a month or so before Mabilat & Courvalin published details of the 'missing links' [157]. The development of a method for the rapid screening of clinical isolates for point-mutations in the TEM gene allowed the identification of TEM-1 and TEM-2 variants that did not necessarily confer a clinically significant phenotype. The technique, called 'oligotyping', probed five loci of the *bla*T genotype with 17-mer oligonucleotides to discriminate point mutations on the basis of a single nucleotide mismatch between oligonucleotide and gene. From the screening of 222 strains expressing a TEM-*type* β-lactamase, nine novel genotypes were identified. Seven of the new β-lactamases were named TEM-13, TEM-14, TEM-15, TEM-16, TEM-17, TEM-18, and TEM-19 [157]. The previously unknown *bla*T-8 and *bla*T-11 genotypes were also identified for the known TEM-8 and TEM-11 phenotypes. Mabilat & Courvalin [157] provided a great deal of information on the molecular epidemiology of the TEM-*derived* β-lactamases, but little information on the biochemical characteristics of the enzymes. A major failing of the oligotyping technique for identification of genotype is that mutations not occurring at the loci probed would not be identified. The clinical isolates that were 'oligoytyped' originated from many different French hospitals, and have remained unavailable for other researchers to examine. The biochemical characteristics of the enzymes described by Mabilat & Courvalin [157] have still not been elucidated. It has remained for other researchers either to create the genotype with recombinant techniques and then study the biochemistry of the enzyme, or to identify examples of the enzymes

from clinical enzymes. Many of the genotypes have now been correlated with a phenotype by one of these two approaches, or been assigned through analogy to other Ambler Class A β-lactamases.

Thus, a strategy for the creation of some of the TEM-*derived* intermediate enzymes by site-directed mutagenesis was designed. The construction of six recombinant enzymes was attempted over a period of nine months. Of these six experiments, four yielded mutants with apparently the correct genotype (TEM-$12_{pUK3002}$, TEM-$2_{pUK3003}$, TEM-$17_{pUK3004}$, and TEM-$7_{pUK3005}$). In the initial strategy these recombinant enzymes were to be used as the basis of further SDM experiments to create more TEM-*derived* intermediate enzymes. All of the β-lactamases created by SDM, except for TEM-17, had been well characterised by other researchers, and the enzymes were available for study. The pUK3004 enzyme did not have the phenotype expected: the pI of the recombinant β-lactamase (pI ~5.5) was lower than expected (pI 5.9 [157, 241]); the rate of hydrolysis of 3GCs was higher than found by other workers for the same point mutation [241]. It was, therefore, concluded that the pUK3004 β-lactamase gene did not encode the TEM-17 β-lactamase. The DNA sequence of *bla*$T_{pUK3004}$ would have to be reassessed in order to categorically define the genotype of the enzyme expressed; unfortunately, the constraints of time prevented such measures. The major criticism of the 'oligotyping' method of Mabilat & Courvalin [157] was that, although probing a 17 to 20-bp region of a specific gene can efficiently detect a point mutation within that region, it does not present any information on mutations of the DNA sequence remote from that site.

I suspect that, as quite commonly occurs with this technique, at least one point mutation was inadvertently inserted into the 750-bp fragment present in the M13 vector during SDM. When the mutant fragment was cloned back into pUK3001 and the β-lactamase expressed, the additional point-mutation(s), in combination with the Glu-104→Lys change, encoded a functional protein with altered biochemical characteristics.

Other researchers had managed to create mutants or identify clinical examples of the missing enzymes, hence as a result of the slow progress of the SDM approach, time was devoted to answering other questions on the evolution of the SHV- and TEM-*derived* β-lactamases.

7. TEM-E2$_{pUK721}$

The first ESBL was identified by Kliebe *et al.* in 1985 [155] and was later shown to be derived from the SHV-1 β-lactamase by a single amino acid mutation [258]. This ESBL (SHV-2) is classified as a Du Bois, Payne & Amyes Group 3 enzyme, and confers high level resistance to the 3GCs. Du Bois, Payne & Amyes Group 2 and 3 β-lactamases all confer significant clinical resistance to the 3GCs. Groups 0 and 1, however, confer low level resistance to the 7-oxyimino cephalosporins which may not cause treatment failure in the clinical setting. Under these conditions the identification of the novel genotype would be missed, and such an omission would have no bearing on the outcome of the antibiotic therapy of the infection concerned. The omission would become a problem if cross-infection of the bacterium expressing the mutant β-lactamase were to occur and subsequent challenge with 3GCs selected a Group 2 or 3 enzyme. Even if treatment failure does occur, partly as the result of a strain expressing a Group 0 or 1 enzyme, the presence of such a β-lactamase may pass unnoticed, as the phenotypes of these enzymes are often similar to those observed for the prototype β-lactamases TEM-1 and TEM-2. Treatment failure may be attributed to other factors, when in reality the mutant β-lactamase is one of, possibly, two or three factors; for example, decreased permeability of the bacterial cell envelope as a result of OMP mutations. Group 0 and Group 1 enzymes often confer low resistance levels and the MICs of the 3GCs for the strains concerned are below the break-points recommended in the BSAC guidelines [196].

Another factor that delayed the identification of ESBL before 1985, especially those conferring low level resistance, was the belief that the development of plasmid-mediated resistance to the 3GCs was impossible. This belief was the result of the information disseminated by the pharmaceutical companies that marketed the 3GCs at this time. Indeed, the consensus belief was that, at the time, these claims were valid. It is unfortunate, but after the introduction of any novel compound, many of the initial claims turn out to be untrue, mainly because of the lack of clinical data on the new drugs.

Payne [141] conducted a retrospective study of ceftazidime resistant isolates stored in the Glaxo Group Research Ltd. culture collection (Greenford, Middx) and identified a TEM-*type* β-lactamase that conferred low level ceftazidime resistance on the host

organism [141, 151]. The strain in which the TEM-E2 β-lactamase was identified was isolated in 1982 and, therefore, pre-dated any other known ESBL by three years. TEM-E2 showed considerable similarity in substrate profile to the recombinant β-lactamase TEM-101 [162], which was created by point-mutation of the arginine-164 codon to code for a serine residue at position 164. Later, Weber *et al.* [189] described a clinical example of a TEM-1-*derived* β-lactamase, designated TEM-12, that encoded the arginine-164→serine substitution which also showed remarkable similarity to the TEM-E2 enzyme. TEM-12 was chromosomally encoded, but was shown to be present on a transposon [259], and only conferred clinically significant resistance to the 3GC ceftazidime in combination with decreased permeability of the outer membrane of the host organism [189]. Furthermore, TEM-E2-*like* mutant β-lactamases could be selected from TEM-1 *in vitro* with ceftazidime [161, 162], hence the evidence strongly suggested that TEM-E2 was derived from TEM-1 by substitution of arginine-164 with serine. The TEM-12 β-lactamase holds a pivotal position in the evolution of the TEM-*derived* ESBL (Figure 4.2 page 179), hence the characterisation of the amino acid sequence of the first known example of an ESBL is essential.

The methodology described in Chapter 3 eventually led to a clear indication that pUK721 encoded two β-lactamases; one with an arginine residue at position 164 and one with a serine residue at position 164. pUK721 was known to produce two β-lactamases, TEM-1 (pI 5.4) and TEM-E2 (pI 5.25) [151], hence as *bla*T-1 encodes for arginine-164, it can be deduced that the second β-lactamase gene encodes for a serine at position 164. No other nucleotide changes were observed from the DNA sequencing gels, therefore it is concluded that the TEM-E2 β-lactamase has an identical genotype to that of TEM-12.

8. ESBL "BACK-MUTATIONS"

The purification of β-lactamases to sufficient purity to be able to determine β-lactamase concentration accurately is a difficult operation. Other researchers have successfully completed the purification and the determination of detailed enzyme kinetics on a number of β-lactamases previously [260, 261], but it was not until Sowek *et al.* [241] published the kinetic profiles of a number of closely related recombinant TEM-1-*derived* β-lactamases that the true relationships became

apparent. The 'Sowek paper' was published in late 1990 and provided the catalyst for the hypothesis that is described here. As mentioned previously, Sowek and colleagues, although presenting a mass of information, failed to realise the full implication of the data. It was not until the data were arranged in an evolutionary context (Figure 4.2 page 179) that the trends became apparent: as capability to hydrolyse the 3GCs increased with a concurrent increase in the number of ESBL mutations, the turnover of the pencillins decreases. The less efficient that a TEM-*derived* β-lactamase is at hydrolysing the early penicillins, the lower the I_{50} value of clavulanic acid becomes, i.e. the more tightly clavulanic acid is bound to the enzyme. It would appear that such a scenario would be evolutionary disadvantageous, especially since the host organisms of ESBL would be challenged with penicillins, or pencillins in combination with the β-lactamase inhibitors in general practice. The Darwinian approach to the evolutionary implications of these findings would explain the relative rarity of the ESBL with respect to the prototype enzymes.

A second finding from the kinetic data was that some of the alternative intermediates, such as TEM-17 and, I suspect, SHV-3, presented no significant selective advantage over the parental enzymes when conferring 3GC resistance. These intermediate enzymes, conversely, did not present a significant disadvantage over the parental species of β-lactamase when conferring resistance to the penicillins (Figure 4.2 page 179). Back-mutation to an alternative intermediate, TEM-17 instead of TEM-12 for example, from TEM-26 would create an enzyme closer to the prototype TEM-1 β-lactamase in its ability to hydrolyse penicillins. This observation would explain the existence of many of the extraneous ESBL that have been identified but, on first inspection, appear to have no evolutionary function.

8.1. THE EFFECT OF AMPICILLIN

In order to test the hypothesis, after repeating the kinetic studies of Sowek and co-workers, the terminus enzyme TEM-5 was passaged against ampicillin at various concentrations. Passage with ampicillin over a short period of time (five days in the first passage experiment) and a relatively long period of time (20 days in the third passage experiment) did not select any backward mutations. No novel β-lactamases were observed as the result of either ampicillin passage.

Comparison of the MICs of the simple penicillins for the various TEM-derivatives reveals that resistance is similar or may actually increase. Other factors, however, influence the MIC values: host organism, gene promoter, plasmid copy number, and permeability barriers [248, 250]. The strains passaged with ampicillin by this method, or any clinical strain challenged with a penicillin, would all be equal in these respects compared to the other bacteria in the culture, if they all harbour the same plasmid. Transfer of plasmid DNA to other organisms, for instance in the gastro-intestinal tract, will probably occur [262], or there may be mutations in the OMPs [189, 248] or the PBPs [263]. This may provide a greater selective advantage than back mutation to an enzyme with a relatively low increase in penicillin hydrolysis compared to the 'terminus' ESBL. The β-lactam antimicrobial agents are known to alter the properties of the bacterial cell envelope at sub-MIC concentrations [264]. The application of a selective pressure with a penicillin was, therefore, not sufficiently severe for a strain harbouring a plasmid encoding a back-mutation. The difference in kinetic profile of a 'terminus' ESBL compared to the prototype β-lactamase, with respect to the early penicillins, does not appear to be a disadvantage. Any organism expressing an SHV- or TEM-*derived* ESBL would confer a level of penicillin resistance that has no clinically significant difference to that conferred by the parental enzymes, TEM-1, TEM-2 or SHV-1.

The effect of plasmid copy number was examined in order to calibrate the MICs of β-lactamases encoded on different clinical plasmids. Although the MICs of the β-lactams tested did increase with copy number, and therefore with β-lactamase expression, the increase is out of proportion with the differences in copy number. A five-ten fold increase in plasmid copy number increased the MIC of the penicillins, ampicillin and amoxycillin, by about three fold but this was quite variable. The MICs of amoxycillin/clavulanic acid, ceftazidime, and cefotaxime are not increased to such an extent with copy number. The MIC of amoxycillin/clavulanic acid, for instance, only doubled for a five to 10 fold increase in copy number. The MICs of ceftazidime and cefotaxime are affected in a similar manner, with the MIC of cefotaxime affected to the least extent of all the β-lactams tested. From this data it can, therefore, be surmised that copy number does influence the MIC of the β-lactams but not by a significant degree. All ESBL identified so far have been encoded by large clinical plasmids [180], which are

invariably present in a low copy number. The effect of the copy number of the clinical plasmids will, therefore, be minimal.

Disney & Dove [174] cloned a number of ESBL into an isogenic expression vector, then determined the MICs of a number of β-lactam antibiotics hosted in two isogenic *E. coli* strains: DCO and DC2 [249]. *E. coli* DC2 is isogenic with strain DC0, except for an *abs* mutation, rendering the strain hyperpermeable to most antibiotics: the effect of permeability of the various β-lactam antibiotics was assessed (see Table 4.2). The MIC values examined in this manner are not subject to variation in plasmid copy number or variability in expression as each TEM ORF is under the regulation of the same promoter, *P3*, from the *bla*T gene of Tn*3*. The MICs of cefuroxime, ceftazidime and cefotaxime decrease concurrently with a

β-Lactamase	*E. coli* strain	MIC (mg/l)‡			
		CFX	CLAV	CAZ	CTX
TEM-1	DC0	8	31	0.5	0.13
	DC2	1	62	0.5	<0.03
TEM-3	DC0	>62	31	62	>62
	DC2	16	62	>62	>62
TEM-5	DC0	>62	31	>62	>62
	DC2	>62	62	62	16
TEM-6	DC0	32	31	>62	16
	DC2	2	62	>62	2
TEM-7	DC0	8	31	>62	2
	DC2	1	62	>62	2
TEM-9	DC0	31	31	>62	62
	DC2	2	62	>62	16

Table 4.2: The effect of outer-membrane permeability on the MIC of various β-lactam antimicrobial agents

‡ Inoculum of 10^4 cfu/spot for each test organism. Abbreviations: CAZ, ceftazidime; CFX, cefuroxime; CLAV, clavulanic acid; CTX, cefotaxime. Data from Disney & Dove (1991) [174].

decrease in permeability for every ESBL examined. The MIC of clavulanic acid is somewhat anomalous, increasing with decreasing permeability.

One conclusion from all the MIC determinations presented is that the technique is not subtle enough to be able to discern the differences in resistance conferred by a group of exceptionally closely related enzymes such as the SHV- and TEM-*derived* ESBL.

8.2. THE EFFECT OF AMOXYCILLIN/CLAVULANIC ACID

Amoxycillin in combination with clavulanic acid, in the ratio 2:1, provides a stronger selective pressure as a result of the joint progressive and competitive nature of the inhibition of the β-lactamase inhibitor [265]. The effect of the clavulanic acid on the ESBL TEM-5 was clearly shown to promote the selection of back mutations. The passage of an *E. coli* strain harbouring TEM-5, at limiting concentrations of amoxycillin/clavulanic acid for five days, promoted back mutation of *bla*T-5 to *bla*T-10, and then *bla*T-12. No TEM-1 variants were observed. Further passage of the mutant plasmid, pUK3007, for 20 days selected strains that either expressed TEM-5 or TEM-1 only. The TEM-5 and TEM-1 β-lactamases represent the two extremes of function of the evolutionary series; TEM-1→TEM-12→TEM-10→TEM-5. A culture, therefore, expressing both TEM-1 and TEM-5 will have the advantages of both enzymes: the broad-spectrum of TEM-5 and the lower β-lactamase inhibitor susceptibility of TEM-1. A strain expressing TEM-5 will be protected against its deficiencies, to some degree, by surrounding strains expressing TEM-1 [2]. The reverse would also be true. The presence of any other intermediate enzyme, for example TEM-12 or TEM-10, would be surplus to requirements. The intermediates are not good at conferring 3GC resistance or low susceptibility to clavulanic acid compared to the prototype TEM-1, or the 'terminus' TEM-5 β-lactamase. The combined effect of expression of the three β-lactamases (TEM-5, TEM-10, and TEM-12) was shown to have a broader range of activity than TEM-5 or TEM-1 alone. The presence of TEM-1 and TEM-5 within a culture would also have a broad spectrum of activity, and only requires the expression of two enzymes rather than three.

Following this hypothesis, the intermediate β-lactamases, TEM-12 and TEM-10, are only expressed during the transition of TEM-5 back to TEM-1. This would explain why only a finite number of strains, after the five day passage with amoxycillin/clavulanic acid, exhibited multiple β-lactamase expression and why strains only expressed TEM-1 or TEM-5 after a further 20 days.

The effect of clavulanic acid on its own needs to be mentioned. Table 4.2 (page 192) illustrates the findings of other workers [30], that the MIC of clavulanic acid is a significant factor at higher concentrations. The MIC of clavulanic acid alone appears to be about 31mg/l [30, 174]; therefore, at higher concentrations of amoxycillin/clavulanic acid (ratio 2:1) the antibacterial action of the clavulanic acid is a contributing factor to the overall MIC. This is why, when determining the MICs of a β-lactam in the presence of a β-lactamase inhibitor, a fixed concentration of the inhibitor is included in the medium: 2mg/l of clavulanic acid with varying β-lactam concentrations. For the purposes of the passage experiment a ratio of amoxycillin and clavulanic acid of 2:1 was employed, as the commercially available preparations of this combination (Augmentin, SmithKline Beecham) are formulated in this ratio. The physiological levels of the two components would, therefore, approximate to a ratio of 2:1.

8.3. *bla*T GENE DUPLICATION

The Southern transfer of plasmid DNA after restriction analysis, and the subsequent probing for the presence of *bla*T genes, revealed that multiple β-lactamase expression within a single strain corresponded to the presence of multiple TEM β-lactamase genes on a single plasmid. This suggests that back-mutation has been facilitated by intramolecular gene duplication. Duplication of the *bla*T-5 gene would, firstly, increase β-lactamase expression. In the second instance, when two β-lactamase genes are present, a point mutation within one of the genes may be selected without loss of the ESBL activity encoded by the TEM-5 gene. Mutations that code for the β-lactamase intermediates would confer a broader-spectrum of activity upon the host organism, hence, present a selective advantage. Further back-mutations would be selected in a similar manner. Whether one of the duplicate genes is deleted prior to a second duplication/mutation event, or one of the two genes has another point mutation selected without deletion, is

not known. The host strain, however, does harbour a heterogeneous population of plasmids encoding a number of combinations of the *bla*T-12, *bla*T-10 and *bla*T-5 genes. Examples of strains that express either combinations of intermediate TEM-*derived* ESBL, or a combination of parental TEM β-lactamase and an intermediate ESBL have been isolated from clinical samples [151, 266]. These reports help to validate the *in vitro* observations.

After the 20 day passage, strains harboured plasmids which expressed either TEM-1 or TEM-5, that were derived from the mutant pUK3007, encoded one or two TEM β-lactamase genes. I suspect that the presence of two *bla*T genes on a single plasmid of a strain expressing only a single β-lactamase was a transitional phase before deletion of one of the identical genes. Hence, if the passage were continued with amoxycillin/clavulanic acid, a heterogeneous population of bacteria would exist that expressed a single β-lactamase, TEM-1 or TEM-5, encoded by plasmids harbouring a single β-lactamase gene.

TRANSPOSITIONAL DUPLICATION OF β-LACTAMASE GENES

The TEM-*derived* β-lactamases have been found on a number of transposons [267, 268], identified as Tn*1*, Tn*2*, Tn*3*, and their relatives. Tn*3* and Tn*21* are class II, or complex, transposons, and represent two of the three related families of this class that have been identified [269]. TEM-1 is usually present on a Tn*3*-like element [180], and some of the enzymes have been found on Tn*21*-like transposons [259]. Studies on Tn*3* have suggested that transposition proceeds via a cointergrate intermediate [270], where replicon fusion facilitates transposition. If the whole transposon harbouring the *bla*T gene is being duplicated under the selective pressure of amoxycillin in combination with clavulanic acid, an alternative method of replication is suggested by my results: the analysis of the mutant plasmid, pUK3007 (~170-kb), showed a duplication of a relatively small region of DNA, about 25-30-kb in size, when compared to the parent pCFF14 (~141-kb). Analysis of the plasmid DNA suggests that transposition occurs via a mechanism of inversion [270]. This mechanism of replication is, however, not regularly associated with class II transposons, implicating that gene duplication may not be the result of transposon duplication.

INTRAMOLECULAR DUPLICATION OF β-LACTAMASE GENES

Another possible method of β-lactamase gene duplication is that the gene itself is duplicated within a transposon. Duplication by this method would probably follow the normal mechanism of resistance gene acquisition employed by class II transposons [111, 269, 271]. The presence of two identical, or nearly identical, genes within the same transposon would somehow interfere with transposition and, therefore, present an evolutionary disadvantage for the transposon. In a stable environment one of the duplicates would be deleted, and the presence of two *bla*T genes on the same transposon would only be present during the transition of an ESBL to a parental enzyme.

This area of the study would be interesting to elucidate further, and probing of the plasmid DNA for specific transposons would help answer some of the new questions raised by these passage experiments.

9. CONCLUSIONS

Conservative mutations, or mutations remote from the active site, do not affect biological parameters in a measurable manner. Evolutionary implications of the single mutations may be relatively minor, as seen in the enzyme TEM-17, and may not be detected during the normal course of events. Unless the resistance profile has been altered some mutations would be difficult to identify. A number of silent mutations, or minimally important mutations, occur frequently with little functional consequence, but prepare bacteria for a more drastic change when challenged further with 7-oxyimino cephalosporins. Substitutions that result in significant changes in the phenotype of the producing organism would not necessarily be observed unless a specific cephalosporin was used during clinical treatment. Most of the first step mutations observed in the TEM β-lactamases would fall into this category; indeed most of them would pass undetected in diagnostic laboratories as they fail to reach the threshold of resistance recommended for break-point sensitivity testing [196]. So the acquisition of a single innocuous mutation may create an enzyme which is poised to confer high levels of resistance after a second, more dangerous mutation.

Although innocuous mutations may exist in the unchallenged population, treatment with a penicillin, as often occurs in general practice, would result in the selection of

the parental enzymes (TEM-1, TEM-2 and SHV-1) over the progeny. Challenge with the 7-oxyimino cephalosporins would select the most resistant β-lactamase in the host population. Only when such an event has occurred will a second mutation event occur to expand the spectrum of the enzyme further. A third mutation may occur during continued treatment with 7-oxyimino cephalosporins to create a more effective extended-spectrum β-lactamase. Cross-infection to other patients, or escape into the environment, may result in the challenge of the host organism with a penicillin and a β-lactamase inhibitor. Such a biological pressure would select against the extended-spectrum β-lactamases and may induce back mutations to the more innocuous enzymes, TEM-17 or SHV-3, for example. Extended-spectrum β-lactamase evolution can be mapped through careful biochemical analysis and the use of molecular biology techniques, and it seems that such evolution of the parent β-lactamase to broad-spectrum activity consists of a series of both forward and backward mutations.

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Outbreak of infection in two UK hospitals caused by a strain of *Klebsiella pneumoniae* resistant to cefotaxime and ceftazidime

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Summary: During an 8-month period, *Klebsiella pneumoniae* resistant to cefotaxime and ceftazidime were isolated from 18 elderly patients in two closely-situated UK hospitals. Amongst these 18 patients, the organisms were isolated from urine samples of 17, from blood cultures of two and from a wound swab of one. The infected patients were located in nine different wards and several of the patients had been transferred between wards, within and between the two hospitals. All the bacterial isolates belonged to serotype K62, were non-typable or reacted only weakly with bacteriophage, showed similar plasmid profiles and were resistant to tetracycline and trimethoprim, thus indicating they were the same strain. Resistance to cefotaxime and ceftazidime was inhibited by clavulanic acid suggesting the involvement of extended-spectrum β-lactamase (ESBL) enzyme activity. This was confirmed by analytical isoelectric focusing, which showed that isolates each produced two β-lactamases with isoelectric points of 7·0 (SHV-3) and 7·6 (SHV-1/2) respectively.

Keywords: *Klebsiella pneumoniae*; resistance to cefotaxime and ceftazidime; extended-spectrum β-lactamase.

Introduction

When the third-generation cephalosporins cefotaxime and ceftazidime were introduced into clinical use in the early 1980s, they offered the advantage of being stable to the then known plasmid-mediated β-lactamases.[1,2] However, there have since been a number of reports of the isolation of bacteria, (in particular *Klebsiella* spp.), which are resistant to third-generation cephalosporins due to the production of extended-spectrum β-lactamases (ESBLs) capable of hydrolysing these compounds.[3,4] Although the earliest known example of an organism resistant to third generation cephalosporins,

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due to production of an ESBL, is a strain of *Klebsiella oxytoca* isolated in Liverpool in 1982,[5] such organisms have presented few significant problems in the UK to date. Early in 1991, a review of resistance to third-generation cephalosporins noted that only 10 UK isolates had been reported in the literature[4] in contrast to the situation in some other countries, particularly France, where a number of hospital outbreaks involving organisms producing ESBLs have been reported.[3,4] Shortly after this review was published, a small outbreak in the Intensive Care Unit of a UK hospital involving eight patients infected or colonized with *K. aerogenes* serotype K16 resistant to third generation cephalosporins, probably due to production of an ESBL, was reported.[6] In addition, with other workers we have recently reported three patients in a London hospital infected with distinct strains of *Klebsiella* spp. resistant to cefotaxime and ceftazidime due to production of ESBLs.[7] In this paper, we report an outbreak of nosocomial infection involving elderly patients in two closely situated UK hospitals which was caused by a cephalosporin-resistant strain of *Klebsiella pneumoniae* producing at least one ESBL.

Methods

Hospital setting

Hospital A is a 600-bed district general hospital in the UK and hospital B is an acute and long-stay hospital for elderly care patients, situated 8 miles away. Hospital A provides the pathology service for patients in hospital B. Elderly care patients are often transferred between the two hospitals.

In November 1990, *Klebsiella pneumoniae* was isolated from the blood of a 74 year-old male patient in hospital A. The patient had been in hospital for 2·5 months during which time he had been nursed on two different wards. The source of the bacteraemia was thought to be the catheterized urinary tract, though this was not confirmed microbiologically at the time. On disc testing (30 μg discs), the isolate showed reduced sensitivity to cefotaxime and ceftazidime. The isolate was referred to the Central Public Health Laboratory (CPHL) where resistance (minimum inhibitory concentration [MIC] $> 16\ mg\ l^{-1}$) to both antimicrobial agents was confirmed. Following this probable urinary tract-associated bacteraemia, cefotaxime was added to the antibiotic set tested routinely against urinary tract isolates from all urines submitted from inpatients in both hospitals. Between January and June 1991, a further 19 isolates of cephalosporin-resistant *K. pneumoniae* recovered from 17 patients were referred to CPHL where they were examined in detail as outlined below.

Antimicrobial susceptibility testing

MIC values for a number of antimicrobial agents were determined by an agar dilution method, using Isosensitest agar (Oxoid) supplemented by 2% (v/v) lysed horse blood (Advanced Protein Products Ltd.), with an

inoculum of 10^4–10^5 organisms per spot. For nine isolates, the MIC values for cefotaxime and ceftazidime were determined with and without the addition of clavulanic acid to the medium at a final concentration of $2\,mg\,l^{-1}$.

Serological and bacteriophage typing
The capsular (K) antigen of each isolate was typed by counter-current immunoelectrophoresis (CIE).[8] Bacteriophage typing was performed as described previously.[9]

Plasmid analysis
Plasmids were extracted according to the method of Kado and Liu[10] and subjected to electrophoresis in 0·8% (w/v) agarose gels.

Attempts to transfer cephalosporin resistance
Attempts were made to transfer cefotaxime resistance from *K. pneumoniae* to *E. coli* strain J62-2 (lac^-rif^r). Equal volumes of broth-grown *K. pneumoniae* and *E.coli* J62-2 (at a concentration of 10^8–10^9 colony forming units [cfu] ml^{-1}) were mixed together and incubated at 37°C overnight. Serial 10-fold dilutions were then plated out on Isosensitest agar containing cefotaxime ($8\,mg\,l^{-1}$) and rifampicin ($50\,mg\,l^{-1}$), and incubated at 37°C for up to 3 days.

β-lactamase identification
Nine isolates were referred to the University of Edinburgh for identification of β-lactamase activity. The β-lactamase enzymes present in sonicated extracts of bacteria were identified by analytical isoelectric focusing.[11]

Results

Description of the outbreak
Between January and June 1991, *K. pneumoniae* organisms resistant to cefotaxime and ceftazidime were isolated from urine (nine catheter specimen samples and eight mid-stream urine samples) from 14 female and three male elderly patients (age range 67–102 years old). Identical organisms were also isolated from the blood of one of the patients and from a wound swab of another. Eleven of the patients had urinary catheters at the time of isolation or had been catheterized previously.

Four of the patients were in three different wards in hospital B at the time that the organisms were isolated, and the others were in hospital A. The relatively insidious and widely spaced nature of the outbreak is shown in Table I. For 16 of the patients, the interval between admission to hospital and the isolation of cephalosporin-resistant *K. pneumoniae* ranged between two and 12 weeks. Two patients, one in a medical ward and one in an elderly care ward in hospital A were found to have cephalosporin-resistant *K.*

Table I. *Patient location and date when* Klebsiella pneumoniae *K62 resistant to cefotaxime and ceftazidime was first isolated*

Hospital	Ward type	Ward	No. of patients in indicated month								Total
			Nov. 90	Dec. 90	Jan. 91	Feb. 91	Mar. 91	Apr. 91	May. 91	June 91	
A	Elderly care	1	1	–	1	–	–	2*	1	1	6
	Elderly care	2	–	–	–	–	1	–	–	–	1
A	Orthopaedic	1	–	–	–	–	–	1†	1	–	2
	Orthopaedic	2	–	–	–	–	–	1	2	–	3
A	Surgical	1	–	–	–	1	–	–	–	–	1
A	Medical	1	–	–	–	–	–	–	–	1	1
B	Elderly care	1	–	–	1	–	–	–	–	–	1
	Elderly care	2	–	–	1	–	1	–	–	–	2
	Elderly care	3	–	–	–	–	–	1	–	–	1
Totals			1	0	3	1	2	5	4	2	18

* One patient had isolates from blood and urine. † Isolates from wound swab and urine.

pneumoniae in urine samples collected within one day of admission. Each of these patients had previously been admitted into the same elderly care ward in hospital A in September and November 1990 respectively. All of the patients from whom cephalosporin-resistant *K. pneumoniae* was isolated had received antibiotics for treatment or prophylaxis following their hospital admission. Twelve patients had received one or more cephalosporins (cefotaxime, ceftazidime or cephradine), 11 patients had received a β-lactam (amoxycillin or flucloxacillin) and three patients had received trimethoprim, a drug to which the organism was also resistant (see below). Three patients had been transferred between the two hospitals during the course of their admission and six patients had been transferred between wards within hospital A.

Antimicrobial susceptibility

All the isolates were resistant to a range of β-lactam antimicrobial agents including ampicillin (MIC > 64 mg l^{-1}), carbenicillin (MIC > 256 mg l^{-1}), piperacillin (MIC > 64 mg l^{-1}), cefuroxime (MIC > 32 mg l^{-1}), cefotaxime (MIC > 16 mg l^{-1}) and ceftazidime (MIC > 16 mg l^{-1}). For nine isolates tested, (six were from patients in five different wards in hospital A and three were from patients in two wards in hospital B), the MIC value for cefotaxime was reduced from 512 mg l^{-1} to 1·0 mg l^{-1} or less in the presence of clavulanic acid, while the MIC value for ceftazidime was reduced from more than 128 mg l^{-1} to 4 mg l^{-1} or less. This observation suggested that resistance to these agents was due to ESBL activity. Analysis of these nine isolates by analytical IEF revealed identical β-lactamase activity, with two bands of pI 7·0 and 7·6 being detected. The former band most probably reflected production of the ESBL SHV-3, while the band with a pI of 7·6 indicated the production of either SHV-1 or the ESBL SHV-2 (the two enzymes have identical pI values). Attempts to transfer cefotaxime

resistance from six of those isolates to *E. coli in vitro* were unsuccessful. All the isolates were additionally resistant to tetracycline (MIC = 8 mg l^{-1}) and trimethoprim (MIC > 8 mg l^{-1}) but susceptible to amikacin (MIC ⩽ 2 mg l^{-1}), gentamicin (MIC ⩽ 1 mg l^{-1}), tobramycin (MIC ⩽ 1 mg l^{-1}) and ciprofloxacin (MIC = ⩽0·25 − 1.0 mg l^{-1}).

Evidence for spread of a single strain

All the isolates exhibited a number of common phenotypic characteristics indicating that they were related epidemiologically. They all belonged to serotype K62, were non-typable or reacted weakly with bacteriophage and had the same antibiogram, as described above. The first 15 isolates referred to CPHL were examined for plasmids and were all found to contain a plasmid of *c.* 100 Md in size, with two of these isolates containing an additional plasmid of 40 Md and two containing two additional plasmids of 40 Md and 2 Md. The two isolates harbouring an additional 40 Md plasmid only were isolated from the first case in November 1990 and from a patient in an elderly care ward in hospital A in January 1991. The two isolates harbouring additional plasmids of 40 Md and 2 Md were isolated from a patient in an elderly care ward in hospital B in March 1991, and from a patient in the orthopaedic ward in hospital A in April 1991, respectively. The latter two patients had not been transferred between wards prior to the date that these organisms were isolated from them.

Discussion

This report describes an outbreak of infection (including two bacteraemias) in two UK hospitals involving *K. pneumoniae* resistant to the third generation cephalosporins cefotaxime and ceftazidime. The mechanism of cephalosporin resistance involved ESBL activity, as shown by inhibition of cephalosporin resistance by clavulanic acid and by IEF analysis of bacterial extracts, which demonstrated the presence of SHV-3 together with a second enzyme which was either SHV-1 or SHV-2.

The evidence for nosocomial transmission of a single strain is that isolates from each of eighteen infected patients belonged to the same serotype (K62), were non-typable or reacted only weakly with bacteriophage, and exhibited identical patterns of susceptibility or resistance to a range of non-β-lactam antimicrobial agents. In addition, each of nine isolates examined exhibited identical β-lactamase activity when analysed by IEF. Although each of the 15 isolates examined harboured a plasmid of *c.* 100 Md in size, there was some variation with regard to the presence or absence of other plasmids, with two isolates harbouring a plasmid of *c.* 40 Md, and two harbouring plasmids of about 40 Md and 2·0 Md. There was no obvious epidemiological link between isolates containing more than one plasmid. Although ESBL activity is frequently encoded on transferable plasmids,[4] repeated attempts to transfer cephalosporin resistance from the outbreak strain to *E. coli in vitro* were unsuccessful. The observation that resistance

to cefotaxime and ceftazidime was not detected in other serotypes of *K. pneumoniae* or in other bacterial species in the hospital suggests that the gene encoding cephalosporin resistance may not be readily transferable either *in vitro* or *in vivo*.

At present the epidemiology of the outbreak has not been defined. Information concerning factors such as faecal or skin carriage by patients and staff is not yet available. It is likely, however, that spread of the strain between a total of 10 wards in two hospitals is related to the frequent transfer of patients between wards. In addition, the observation that all the patients from whom the strain was isolated had received one or more courses of antimicrobial agents to which the strain was resistant, suggests that usage of such agents may provide a selective pressure for the spread of such strains. Although detailed epidemiological data are not available, it is reasonable to assume, for the time being, that the underlying epidemiology of the outbreak is likely to resemble that suggested previously for nosocomial *Klebsiella* spp.[12]

Attempts to control the outbreak included re-inforcement of standard infection control procedures. In addition, clinicians were advised to avoid monotherapy with injectable cephalosporins in the empirical therapy of bacteraemia pending microbiological confirmation. Selective digestive tract decontamination, as reported by Taylor and Oppenheim[6] in relation to an ITU outbreak is not a suitable option for relatively long-stay elderly care patients.

The origin of the strain of *K. pneumoniae* causing the present outbreak is not known. Organisms of serotype K62 are relatively uncommon in the UK comprising only 3·5% of strains of *K. pneumoniae* submitted to CPHL for typing (B. Ayling-Smith, unpublished). A strain of *K. pneumoniae* K62 resistant to cephalosporins has been described in a hospital in Ghent, Belgium but differed from the strain described here in that it did not produce the ESBL SHV-3, but produced either an enzyme with a pI of 6·5 designated CAZ-hi or an enzyme with a pI of 7·5 designated FUR-2.[13] A strain of *K. pneumoniae* producing SHV-3 has been described in a hospital in Paris, but the serotype of the strain was not reported.[14] Irrespective of the origin of the strain described in the present report, microbiologists and clinicians need to be aware that cephalosporin-resistant *K. pneumoniae* capable of intra- and inter-hospital spread is now to be found in the UK. Elderly care patients, intermittently or continuously catheterized for urinary drainage and who are often transferred between wards and hospitals may be a special risk group for the acquisition and dissemination of such organisms.

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words such as "You can be sure that I will be giving you all the results as soon as I receive them and I will tell you only the truth".

Truth leads to trust. Touching leads to trust. Trust is never more critical than in the optimum managment of patients who have had to be given bad news. I would also emphasise the importance of never withholding the bad news from any mentally competent adult, no matter what advice may be received from relatives or others.

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Compliance and tuberculosis treatment

SIR,—Your April 6 editorial (p 823) states that "Doctors no longer have the right—if, indeed, they ever had—to expect patients to comply with their advice, whether proscriptive or prescriptive". We strongly support this tenet; however, we suggest one major exception. Tuberculosis is spread almost exclusively by human-to-human aerogenic transmission: when a patient with pulmonary disease coughs, he generates an aerosol containing tubercle bacilli which may then be inhaled by others. We contend that doctors have not only the right but also the obligation to see that the patient adheres to the prescription (chemotherapy) or the proscription (quarantine). On the premise that the tubercular patient potentially infringes the right of a citizen to be free of disease, laws throughout the US strictly constrain public access for patients with communicable (untreated) pulmonary tuberculosis. Failure on the part of physicians to be assertive in ensuring regular administration of antituberculosis medications has dire consequences: high rates of treatment failure/relapse, continued contagion, and acquired drug resistance.

With the closure of sanatoria and the abolition of most specialised tuberculosis clinics, tuberculosis treatment in North America and Europe has been "mainstreamed" into the general health care system over the past three decades. Unfortunately, during this era non-compliance has become the major impediment to the successful treatment of these patients.[1] A major element in this trend has been the laisser-faire policy represented by your editorial position on the care of these patients. Medication cannot be forced upon unwilling patients by the practitioner or the state; however, aggressive, large-scale programmes of directly observed or supervised therapy should be used for those proven to be or at high risk of being non-compliant with therapy. Every effort should be made to induce adherence to treatment—and if this cannot be accomplished and the patient(s) remain contagious, public agencies should be willing to quarantine such individuals. Although this always has been a defensible proposition, it is even more compelling in an era of HIV which has seen a substantial increase in tuberculosis case rates in many regions, including epidemics of multiply resistant disease in several locations in the United States.

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Frequency of rhodopsin codon 23 mutation and retinitis pigmentosa

SIR,—Although those of us with an interest in retinitis pigmentosa (RP) welcome Dr Sorscher and Dr Huang's description (May 11, p 1115) of a rapid method to detect a particular rhodopsin mutation, we feel that they make several misleading statements that should be clarified. Firstly, they state that the frequency of autosomal dominant RP (ADRP) in the general population is "about 1 in 3500". The prevalence of all types of RP is indeed in this region, but the ADRP subgroup represents only 19–26% of the total, which includes autosomal recessive and X-linked forms as well as a sizeable sporadic group.[1] Secondly, Sorscher and Huang ambiguously state that the rhodopsin codon 23 mutation was seen in 17 of 148 individuals "with the disease"—referring to a series of unrelated ADRP patients of North American origin.[2] It should be noted, however, that this point mutation was not found in ninety-one unrelated European ADRP families[3] and that many other rhodopsin mutations have since been found in ADRP families.[4,5] A large number of ADRP families are unlinked to this region and therefore represent mutation(s) at other remote gene(s).[6,7]

We welcome the ingenious methods that Sorscher and Huang present for mutation detection, but they have unintentionally inflated both the frequency of ADRP and of the rhodopsin codon 23 mutation, which is responsible for the disease in a small proportion of RP patients.

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Ceftazidime-resistant *Klebsiella pneumoniae*

SIR,—The incidence of Enterobacteriaceae with resistance to third-generation cephalosporins due to plasmid-mediated, expanded-spectrum β-lactamases (ESBL) may be underestimated. We report here three ceftazidime-resistant *Klebsiella pneumoniae* strains isolated in a London hospital during 1990.

Case 1 (16, F; Guillain-Barré syndrome). She had been transferred from an Athens hospital. Her urine grew *K pneumoniae* (serotype K69) but antibiotics were not required during her 2-month hospital stay in London. Minimum inhibitory concentrations (MICs) were estimated on 'Isosensitest' agar with an inoculum of 10^4 colony-forming units and aerobic incubation for 18 h. An MIC above 2 mg/l is taken as indicating resistance to ceftazidime.[1] The MIC of ceftazidime was 64 mg/l, falling to 0·25 mg/l on addition of 2 mg/l clavulanic acid, which inhibits ESBL.

Case 2 (28, M; quadriplegia). He was admitted with urinary retention and fever. *K pneumoniae* (non-typable) was isolated from both urine and blood. The MIC of ceftazidime was 512 mg/l, reduced to 4 mg/l by clavulanic acid. The strain was also resistant to gentamicin and netilmicin. He was successfully treated with intravenous amikacin after a lack of response to 24 h of intravenous cefotaxime 2 g three times daily. He had been an inpatient in two other London hospitals in the previous 5 years, with no history of foreign travel.

Case 3 (17, M; neutropenia after bone-marrow transplantation for acute myeloid leukaemia). He became febrile a week after the graft (day 1) and the fever persisted despite empirical antibiotic therapy. *K pneumoniae* was isolated from blood culture on days 8, 12, and 15. By day 15 the MIC of ceftazidime was 32 mg/l, reduced by clavulanic acid to 1 mg/l. Imipenem 500 mg four times a day was started on day 15, the organism being sensitive by Stokes' method, and he became apyrexial within 24 hours. On day 21 fever returned, with profound hypotension, and *K pneumoniae* was again isolated from blood culture. The MIC of ceftazidime was now 512 mg/l, reduced to 4 mg/l by clavulanic acid. Doubling the dose of imipenem resulted in a second defervescence and a slow clinical recovery. All isolates were serotype K48.

All patients were nursed in isolation and no secondary cases occurred. The klebsiella strains showed resistance to cefotaxime and aztreonam, and up to five further classes of antibiotics. Resistance to third-generation cephalosporins was successfully transferred to *Escherichia coli* J62 from the *K pneumoniae* strains from the first two cases, consistent with these ESBLs being plasmid-mediated; and plasmids of 60 and 70 MD, respectively, were found on agarose gel electrophoresis.

These three cases illustrate the possible origins of organisms producing ESBLs—namely, international transfer with the patient, inter-hospital transfer, and selection of the resistant strain within a hospital. Nosocomial outbreaks of ESBL-producing Enterobacteriaceae with multiple resistance[2] to a range of antibiotic classes have occurred in Europe[3,4] and the USA.[5] The continued use of third-generation cephalosporins exerts substantial selection pressure in favour of ESBL-producing organisms and also for the evolution[6] of ESBLs. This, together with the introduction of similar oral cephalosporins, such as cefixime, into the community, should prompt laboratory surveillance for such strains. We suggest a variation of the double-disc synergy test[3] on isolates showing moderate resistance to cefotaxime. Should the use of third-generation cephalosporins be restricted?[7]

We thank Dr T. L. Pitt, Central Public Health Laboratory, for serotyping the isolates, and Prof J. M. T. Hamilton-Miller and Dr S. H. Gillespie for advice and comments.

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In-vivo monitoring of neuronal loss in Creutzfeldt-Jakob disease by proton magnetic resonance spectroscopy

SIR,—The advent of bovine spongiform encephalopathy has renewed interest in novel approaches to the clinical diagnosis of spongiform encephalopathies such as Creutzfeldt-Jakob disease (CJD). We have seen a case of sporadic CJD where the only magnetic resonance imaging abnormalities were mild cortical atrophy and hyperintensities in the lentiform nuclei, as noted previously.[1] By contrast, proton magnetic resonance spectroscopy (MRS) demonstrated significant metabolic alterations in cortical grey matter and white matter and in striatum. Right frontal lobe biopsy, done 2 days after these scans, revealed fine vacuolation of the neuropil (spongiform change) in the deep grey-matter with a slight loss of neurons and gliosis, consistent with CJD.

After a cold in August, 1990, a 53-year-old woman had difficulty sleeping and complained of dizziness. When cycling she found it hard to keep her balance. A neurologist noted trunk and gait ataxia and a tremor of her right thumb. By October she had become forgetful, had difficulty expressing herself, and her speech was slurred. Nocturnal anxiety and paraspasticity ensued and she was admitted to hospital in November. Her ataxia worsened as did her memory. Paranoid-hallucinatory states required treatment with haloperidol for a week. In December a progressive left-sided spasticity developed. Dysarthria further impeded her speech and she became demented. CSF analysis revealed oligoclonal banding. EEGs revealed diffuse slow and α-range complexes with temporo-occipital sharp wave θ and δ-complexes. No 1–2 Hz periodic sharp wave complexes (PSWC) were seen. In February, 1991, the patient was in a coma and showed spontaneous myoclonus, predominantly in her right arm. An EEG showed PSWCs, supporting the diagnosis of CJD.[2] In March localised proton MRS was done with 20 ms echo-time stimulated-echo sequences (2·0 T Siemens 'Magnetom').[3] Measuring times were 6·5 min with 128 accumulations and repetition times of 3 s.

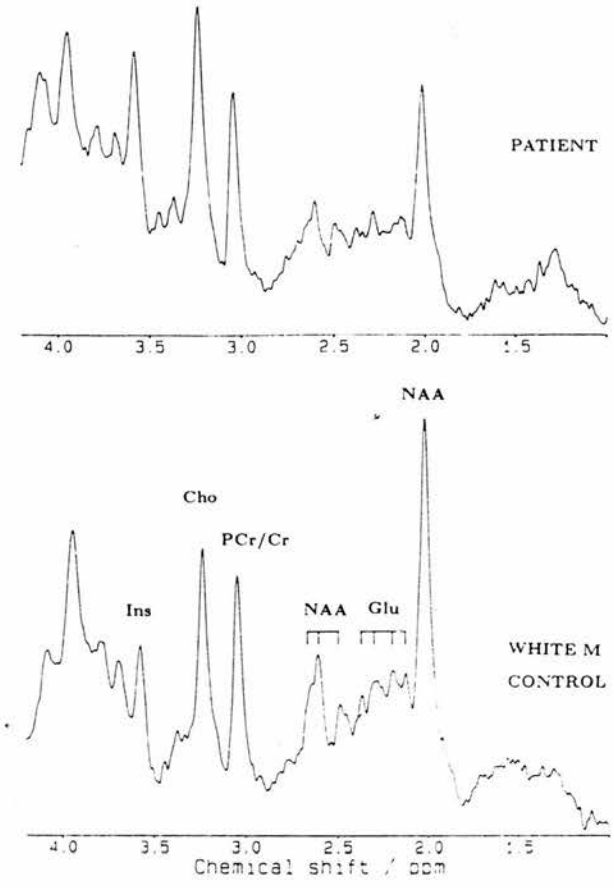

Fig 1—Localised proton MR spectra of parietal white-matter.

Upper: Patient with CJD, exhibiting reduction of NAA and PCr/Cr and increase in Ins.
Lower: control.
Major metabolites include N-acetylaspartate (NAA), glutamate (Glu), creatine (Cr), and phosphocreatine (PCr), choline-containing compounds (Cho), and myo-inositol (Ins). Volume of interest 12 ml.

Fig 1 shows the proton MR spectrum of the left parietal white-matter of the patient, compared with that of a 56-year-old healthy female control. The N-acetylaspartate (NAA) peak was reduced (40%), as was the creatine/phosphocreatine ratio (10%); there was an increase in inositol (30%). Similar findings were observed contralaterally and in frontal white-matter except for slightly lower values of choline-containing compounds. Two volumes of interest in predominantly grey-matter were investigated in paramedian frontal and parietal locations; only NAA was diminished (30%) (fig 2). MRS in the hyperintense region of the striatum showed a fourfold loss of metabolites. The remaining metabolite pattern exhibited a further reduction of NAA relative to creatines and cholines.

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Can clavulanic acid reverse extended-spectrum β-lactamase mutations?

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The SHV- and TEM-*derived* extended-spectrum β-lactamases (ESBL), have emerged as a response to the clinical use of the third-generation cephalosporins (3GCs), and have become an enormous clinical threat to this class of pharmaceuticals. *Escherichia coli* J62-2 (TEM-5_{pCFF14}) was cultured in liquid media containing limiting concentrations of amoxycillin and clavulanic acid (ratio 2:1) (co-amoxiclav). The cultures were sub-cultured five times into fresh media containing the same antibiotics. Ten colonies were purified from each sub-culture. Minimum inhibitory concentrations (MICs) of ampicillin, amoxycillin, ceftazidime, cefotaxime and co-amoxiclav revealed a few strains with increased resistance to co-amoxiclav, ceftazidime and cefotaxime. Isoelectric focusing demonstrated that the increased resistance corresponded with multiple β-lactamase expression (pI 5.57, 5.5 & 5.25). Electrophoresis of plasmid DNA confirmed that each organism contained a single plasmid, of similar size to pCFF14 (150-kb). Analysis of enzyme kinetics of each β-lactamase compared to TEM-1 (pI 5.4), TEM-5 (pI 5.5), TEM-10 (pI 5.57) and TEM-12 (pI 5.25) suggested that the unknown β-lactamases were TEM-5, TEM-10 and TEM-12. Southern blotting of restricted plasmid DNA showed gene duplication in the mutant plasmid (pUK3007) when compared to the parent plasmid (pCFF14). *E. coli* J62-2 (pUK3007) was cultured in liquid media containing limiting concentrations of co-amoxiclav. The cultures were sub-cultured 20 times into fresh media containing the same antibiotics. Analysis of strains revealed that anomalous MICs of co-amoxiclav, cefotaxime and ceftazidime corresponded to the presence of two one of two β-lactamases (pI 5.5 & 5.4). Plasmid DNA was of similar size to pUK3007, and Southern blotting showed the presence of one or two TEM β-lactamase genes. Enzyme kinetics of each β-lactamase suggested that the unknown enzymes were TEM-5 and TEM-1. Our results suggest that clavulanic acid, in combination with amoxycillin, selects back mutations from ESBL to TEM-1 by a mechanism of gene duplication.

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REVIEW ARTICLE

Antibiotic resistance in bacteria

—a review based on a symposium held on 5 January 1991 at Addenbrookes Hospital, Cambridge and chaired by Professor J. T. Smith

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Introduction

Antibiotic resistance tends to be discussed by groups who work on related antibiotics. The symposium, which provided the basis for this review, was a rare opportunity for scientists working on quite different aspects of resistance to present and discuss their results. Much emphasis has been placed on the mechanisms of resistance and the first five papers reflect this. They demonstrate the quite different resistance problems encountered with each class of antibacterial drug. The remaining four papers take a different, and often less expressed approach, examining the impact that resistance has on clinical populations and our ability to treat the patient. These papers examine the effect that resistance has on the virulence and pathogenicity of bacteria and how the host is affected by resistant strains. This section ends with views about the future of antibiotics.

Resistance to antifoliate antibacterial agents

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The folate pathway occupies a central metabolic role leading to protein and nucleic acid synthesis (fig. 1). Two types of antifolate agent have gained wide acceptance and are of clinical importance: (i) the sulphonamides, that inhibit the bacterial enzyme dihydropteroate synthetase (DPS), which catalyses the reaction of *p*-aminobenzoic acid (PABA) with a pteridine derivative and are selectively toxic for bacteria because man does not require this enzyme; (ii) trimethoprim, that inhibits the ubiquitous enzyme dihydrofolate reductase (DHFR), and is selectively toxic because its affinity for the bacterial enzyme is some 10 000-fold greater than that for the mammalian enzyme. Following its initial introduction, trimethoprim was prescribed originally only in combination with a sulphonamide, usually sulphamethoxazole, for reasons which, at the time, seemed justified, but upon which, for many applications, substantial doubt was subsequently cast.[1,2] In this paper, acquired resistance to sulphonamides and trimethoprim is summarised with particular reference to recent developments in molecular biology which enhance our understanding of resistance to these important antibiotics.

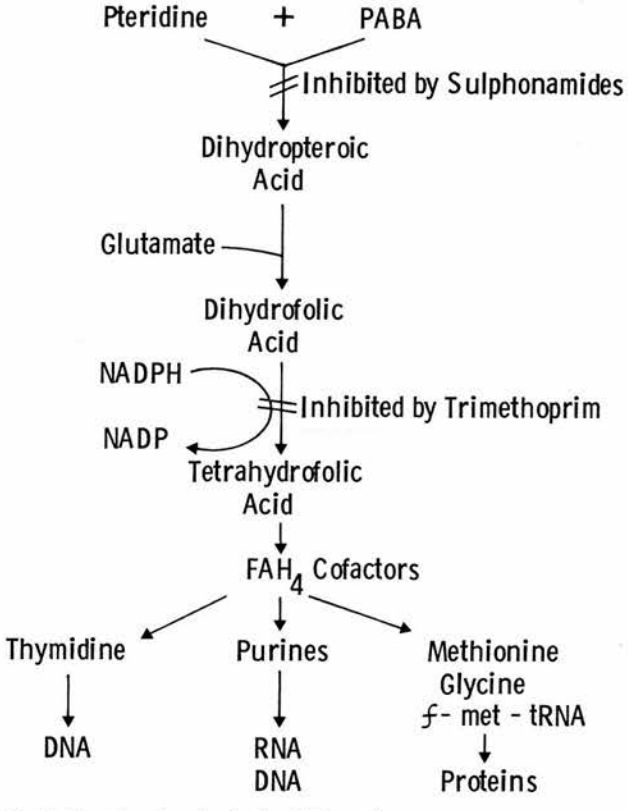

Fig. 1. Reactions involve in the folate pathway.

Incidence of resistance

Although a prime requirement is to distinguish between intrinsic and acquired resistance, other difficulties have been caused in the past by the use of different definitions for the term "resistant". In addition to the fact that clinically relevant resistance is not the same as laboratory-determined resistance, particular problems arise in susceptibility to sulphonamides and trimethoprim in relation to choice of resistance breakpoints, inoculum size and the correct medium.[3] Other local factors, such as the occurrence of epidemic strains, or selection and choice of particular specimens and patients, may greatly influence the "incidence" of resistant strains. Therefore, assessments of changes

number is inadvertently used by different investigators for different enzymes or genes. Gene probes for each of the existing recognised DHFR types can be supplied so that investigators can ensure that they are not duplicating previous work. We would also request that when each investigator is ready to confirm the identification of a new gene, a copy of the new gene, or the appropriate probe, should be donated for future distribution. By this approach, although DHFR type numbers will gradually less reflect biochemical properties, we can ensure that duplication will not occur and DHFR types will be correctly placed in the classification scheme.

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Table I. Incidence of resistance to sulphonamides (Su) or trimethoprim (Tp), or both, among isolates of enterobacteria causing urinary tract infection from the Nottingham area

Species		Percent of isolates that were			Total per cent that were	
		Su^RTp^S	Su^RTp^R	Su^STp^R	Su^R	Tp^R
E. coli	Hosp.	20·2	14·3	5·1	34·5	19·4
	Comm.	18·3	13·3	4·3	31·6	17·6
Klebsiella/Enterobacter spp.	Hosp.	7·6	10·2	5·7	17·8	15·9
	Comm.	9·8	11·0	8·2	20·8	19·2
Proteus spp.	Hosp.	4·0	7·7	14·3	11·7	22·0
	Comm.	3·7	6·3	15·5	10·0	21·8

R, resistant; S, sensitive.
* Resistance was defined as described previously,[7] with breakpoints of trimethoprim lactate 8 μg/ml and sulphamethoxazole 16 μg/ml.

in resistance are more significant if they are made from long-term follow-up surveys in a single centre, and several such surveys have been performed with isolates of Enterobacteriaceae from different parts of the world.[4]

Table I summarises the latest available data (1989) on the incidence of resistance to sulphonamides and trimethoprim among isolates of Enterobacteriaceae causing urinary infection from the Nottingham area. The incidence of sulphonamide resistance has remained stable for many years, but the incidence of resistance to trimethoprim has increased considerably since surveillance commenced in 1978.[5] With the exception of localised outbreaks of infection with resistant strains, comparable incidences of resistance in Enterobacteriaceae are also found generally in other developed countries.[4] In contrast, the incidence of resistance to sulphonamides or trimethoprim, or both, in developing countries tends to be considerably higher (30–60% in general hospital isolates of *Escherichia coli*).[4,6] These higher incidences probably reflect the epidemic spread of particular resistant strains, plasmids or transposons in response to the continuous selection pressure imposed by the careless or unregulated use of trimethoprim and other antibiotics.

Table I also provides an interesting illustration of the effect of changes in selection pressure. Trimethoprim was, originally, prescribed always in combination with a sulphonamide. Consequently, all strains resistant to trimethoprim were also resistant to sulphonamides. Trimethoprim became available for use alone in the UK in 1979 and, since then, the proportion of strains resistant to trimethoprim and susceptible to sulphonamides has increased steadily[7] to the levels shown in table I. In the light of earlier controversy,[1,2] it is interesting, although rather academic, to speculate as to whether the same overall incidence of trimethoprim resistance in Enterobacteriaceae would have been reached so rapidly had trimethoprim continued to be prescribed only in combination with a sulphonamide.

The situation in gram-positive bacteria is somewhat different. Although resistance to sulphonamides has been known for many years, the incidence of acquired trimethoprim resistance remained negligible until the advent of methicillin-resistant staphylococci (MRS) in the early 1980s. A considerable number of MRS are resistant also in trimethoprim,[4] but the overall frequency of strains with resistance to both methicillin and trimethoprim varies widely among strains of different origin, depending largely on the occurrence of outbreaks of infection involving particular resistant strains, but is generally low.[4]

Mechanisms of resistance to sulphonamides

Resistance to sulphonamides may be encoded either by the chromosome or by plasmids. Chromosomal mutations leading to hyper-production of PABA occur and these overcome the metabolic block imposed by the inhibition of DPS by sulphonamides, but they seem to be of relatively minor clinical importance. The commonest mechanism of resistance is the production of an altered form of DPS for which sulphonamides have a reduced affinity. Although chromosomal mutations leading to the production of such altered enzymes may occur, most clinically significant sulphonamide resistance is associated with the production of altered forms of DPS encoded by antibiotic resistance plasmids.

Two distinct types of DPS (I and II) encoded by plasmids have been characterised in gram-negative bacteria; DPS-I (encoded by the gene *sulI*), has only 57% nucleotide sequence homology with DPS-II (encoded by *sulII*), and their deduced polypeptides show only *c.* 50% similarity.[8] The *sulI* gene is often located on transposons related to Tn21, or on large self-transmissible plasmids that show similarities to Tn21 in the resistance region.[9] In contrast, the *sulII* gene not only encodes a different enzyme, but is associated with different types of plasmids. The DPS-II enzyme is somewhat more stable in a cell-free extract than DPS-I, and the *sulII* gene is carried mainly on small non-conjugative plasmids.[8,10] Both of these plasmid-encoded enzymes can be distinguished readily from the resistant DPS encoded by the *sulA* gene on the chromosome of *Streptococcus pneumoniae*,[11] with 43% DNA similarly between *sulA* and *sulI* or *sulII*, respectively.[8]

Mechanisms of resistance to trimethoprim

Acquired resistance to trimethoprim may also be encoded by the chromosome or by antiobiotic resistance plasmids. There is in-vitro evidence that trimethoprim can select for chromosomal mutants of pathogenic bacteria that lack the enzyme thymidylate synthetase.[11] Such "thy" mutants have lost the normal ability to synthesise thymidylate and, in order to grow, require exogenous supplies of thymine or thymidine to enable them to synthesise thymidylate via the "salvage" pathway.[12] The action of trimethoprim would be adversely affected by the presence of such exogenous supplies under pathological conditions, but very few clinical isolates with this mechanism of resistance have been reported,[3] perhaps because of failure to recognise them.

An alternative type of resistance is observed when mutations lead to overproduction of the normal chromosomally encoded DHFR. Mutants of this type have been obtained from several gram-positive and gram-negative species *in vitro*,[3] but, again, seem to be relatively rare among clinical isolates.[13] Impermeability mutants have also been reported in a various genera, and are of particular interest because of the reported cross-resistance between trimethoprim and other antibiotics such as the quinolones.[14]

However, as with the sulphonamides, the commonest mechanism of acquired resistance to trimethoprim results from the production of an insusceptible target enzyme. Mutant forms of the normal susceptible DHFR encoded by the chromosome have been found occasionally in clinical isolates,[13] but by far the most important and commonest mechanism of resistance is associated with the production of an additional, insusceptible, form of DHFR encoded by genes located on self-transmissible or mobilisable plasmids and transposons.[4] The remainder of this section is devoted to a brief description of these enzymes and the possible evolutionary relationships between them.

Plasmid-mediated trimethoprim resistance was dominated initially by the spread of genes encoding DHFR types I and II, but seven major groups (types I–VII) of DHFRs insusceptible to trimethoprim have now been characterised in gram-negative bacteria. Several of these major groups are further divided into subtypes, and an additional insusceptible DHFR (type S1) has been characterised in staphylococci. The identification of each of these enzyme types on the basis of biochemical and biophysical properties has been reviewed previously[4] and, as with the plasmid-encoded β-lactamases, further DHFR types probably await discovery. Indeed, it should be noted that two DHFRs isolated independently in the UK and Scandinavia have both been designated "type VII".[15,16] The remainder of this article refers to the UK enzyme, but further adjustments in nomenclature may be required when the precise relationship between these two enzymes, and the other DHFRs characterised previously, has been investigated fully.

The time-consuming nature of biochemical identification, involving the extraction and purification of each individual enzyme, has encouraged the development of a set of DNA probes which are capable of distinguishing between the genes encoding the different enzymes. These probes are particularly useful for epidemiological studies[17] and are available currently for DHFR groups I–V, VII and S1.[4,18,19] When used in combination with high stringency wash conditions, the probe set is capable of distinguishing clearly between the major enzyme groups. One anomaly concerns DHFR group III, which had originally been thought to contain three directly related subtypes—IIIa, IIIb and IIIc. Each subtype has similar biochemical properties, albeit with markedly different iso-electric points, and confers only a moderate level of trimethoprim resistance.[4] However, no hybridisation has been detected between the standard group III probe (constructed from the type IIIa gene) and the genes encoding types IIIb and IIIc (C. J. Thomson and S. G. B. Amyes, personal communication). Therefore, it seems probable that enzymes belonging to these subtypes, although similar biochemically, may have evolved independently (see below).

Further investigations of the possible evolutionary relationships between the different DHFR groups have been stimulated, in part, by observations that some of the group-specific DHFR probes can, when used under conditions of reduced stringency, react with genes encoding enzymes belonging to other DHFR groups.[4] Several of the enzymes and genes involved have now been sequenced, with particular attention being paid to the amino terminal (N-terminal) region which forms most of the active site for all DHFRs.

The two subtypes within group I (Ia and Ib) share 71% amino acid homology and 65% nucleic acid homology with each other (H.-K. Young, personal communication), but only 29% amino-acid homology with the susceptible chromosomal DHFR.[4]

The three subtypes within group II (IIa, IIb and IIc) share extensive (>78%) amino-acid sequence homology with each other,[4] but are quite unlike any other plasmid or chromosomal DHFR; it has been postulated that they may be derived from an oxidoreductase which acted originally on a completely different substrate.

The type IIIa DHFR shares 51% homology with the *E. coli* chromosomal enzyme, perhaps suggesting that they may have shared a common evolutionary origin.[4] In contrast, the type IIIb enzyme is clearly distinct from the type IIIa enzyme, sharing only 15 of the first 47 amino acids in the N-terminal sequence, and differs also from the *E. coli* chromosomal enzyme and the types I, II, V and S1.[20] These observations support the hypothesis, referred to above, that the subtypes IIIa and IIIb, although biochemically similar, have evolved independently.

The type IV enzyme, like subtype IIIa, shows most homology (40%) with the *E. coli* chromosomal enzyme, but is clearly distinct from the other plasmid-encoded DHFRs.[21]

The type V enzyme, in contrast, is closely related to the types Ia[9] and Ib (H.-K. Young, personal communication), with 75% and 87% homology respectively. There is a similar close relationship between the type VI enzyme and types Ia (63% homology) and V (61% homology).[22] Sequencing results for the type VII enzyme are not available yet, but hybridisation experiments have indicated a close relationship between the the V and VII genes.[19]

All of the plasmid-encoded DHFRs from gram-negative bacteria seem to be unrelated to DHFR type S1 from gram-positive bacteria.[4]

Envoi

Genes encoding resistance to the main antifolate antibacterial agents, sulphonamides and trimethoprim, are now distributed widely among bacteria in most environments. The precise incidence of resistance in local ecosystems is probably related closely to the extent of the selection pressure exerted by local usage of antibiotics. Most clinically significant resistance to antifolates is associated with plasmid-carried genes, often transposable, which encode alternative drug-insusceptible target enzymes. Two major types of plasmid-encoded DPS conferring resistance to sulphonamides have been characterised, and trimethoprim resistance is conferred by at least seven major groups of plasmid-encoded DHFR in gram-negative bacteria, with an additional plasmid-encoded DHFR in gram-positive bacteria. Among the gram-negative DHFRs, groups I, V, VI and VII seem to be closely related, groups IIIa and IV show some similarities to trimethoprim-susceptible chromosomally-encoded enzymes, and group II enzymes seem to be completely distinct from all other types. The combined use of biochemical and molecular biological techniques has enabled the inter-relationships between the different genes and enzymes to be investigated, and is now providing us with an insight into the evolutionary mechanisms underlying the emergence of resistance to the antifolate antibacterials, and perhaps to antibiotics in general.

PLASMID-MEDIATED β-LACTAMASES RESPONSIBLE FOR PENICILLIN AND CEPHALOSPORIN RESISTANCE

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Alexander Fleming's major contribution to medical science was the recognition of the potential of pencillin. He could not have known that he had stumbled upon a group of antibiotics that were not only going to prove to be the most extensively used antibacterial agents throughout the world but also prove to be the most durable. As with almost every other group of antibacterial drugs, the emergence of bacterial

resistance soon threatened their future value. The 50th anniversary of Abraham and Chain's first discovery of an enzyme capable of destroying penicillin,[23] a β-lactamase, has just passed and there is still much debate as to whether β-lactamases really do compromise our reliance on this group of drugs.

If we had had only pencillin G, this battle may have been lost. The strategy used to overcome β-lactamase attack was to modify the antibiotic so that it was no longer capable of fitting into the active site of the enzyme. This proved successful for a period with the β-lactamases produced by staphylococci. The ability to prepare 6-amino penicillanic acid and substitute functional groups, which do not allow the binding of the antibiotic at the active site of the enzyme, to produce methicillin and the oxacillins, kept the staphylococci under control for more than 10 years. However, the adaptability of the β-lactamases and the acquisition of a gene capable of changing the penicillin binding protein (PBP) 2, has now put many staphylococci beyond the reach of β-lactams. Neu demonstrated that, in the developed world, more than 95% of nosocomial staphylococci possessed β-lactamases.[24]

The substitution of functional groups on 6-amino penicillanic acid led to the development of ampicillin, amoxycillin and carbenicillin and allowed the large Gram-negative rods to be controlled by penicillins. The widespread use of ampicillin resulted in the emergence of resistance in 1962. Anderson and Datta[25] showed that resistance to ampicillin could be plasmid-mediated and thus became freely transferable from one species to another. However, little distinction was made between plasmid and chromosomally-determined β-lactamases. In 1973, Richmond and Sykes divided all β-lactamases into five groups based on the substrate profile and response to inhibitors.[26] The substrate profiles were, at best, V_{max} determinations and often were merely determinations of rates of hydrolysis at a single fixed substrate concentration. This value was usually expressed relative to that obtained with a standard β-lactam substrate, i.e., penicillin G, ampicillin, or cephaloridine. The rate of hydrolysis allowed a preliminary classification into oxacillinases, carbenicillinases or broad-spectrum drugs. However, this method of classification had a number of drawbacks. It did not take account of the affinity of the β-lactamase for the substrate (i.e., the K_m) nor did it consider amino-acid sequence homology. It classified all plasmid encoded β-lactamases into just two of the groups, based on the inhibitory activity of cloxacillin.

Ambler[27] introduced a scheme based on sequence homology of the amino acids. He identified two classes of enzyme—Class A enzymes have a serine group at their active site and have a mol. wt of *c.* 29 000. They are either penicillinases or broad spectrum. These enzymes show considerable homology with D-alanine carboxypeptidase and may have a common evolutionary origin. Both chromosomal and plasmid-determined enzymes fall into the group. Class B enzymes include metallo-enzymes and have no amino acid homology with Class A enzymes. Class C was added by Jaurin and Grundstrom,[28] to include β-lactamases with serine involved in the active site but very different in structure from Class A proteins. These enzymes determine cephalosporin resistance, showing no sequence homology with Class A enzymes. Huovinen *et al.*, having sequenced the enzyme PSE-2[29] and compared its structure with the OXA-2 β-lactamase,[30] proposed a fourth class D; enzymes of quite distinct substrate specificities had related structures. The final class, E, was created for the β-lactamases from *Xanthomonas maltophilia*, metallo-enzymes like Class B but with no sequence homology with Class B.[31] This scheme again classified all plasmid-encoded β-lactamases into two groups, which were still very broad. Indeed, some of these enzymes within a class show less than 40% homology with other β-lactamases.

Huletsky *et al.*[32] recently reported the phylogenetic relationships between the Class A β-lactamases that have been sequenced. His cladogram showed a vast diversity in amino acid structure, with as little as 35% homology, although the basic shape of the molecule is conserved. Whereas this scheme may indicate how related enzymes may be, it cannot be extended to allow comparison of β-lactamases in the clinical situation. This is demonstrated with three enzymes in this group, TEM-1, TEM-3 and SHV-1. TEM-1 and SHV-1 are very similar biochemically; they hydrolyse penicillins and early cephalosporins, but not later cephalosporins. However, they are phylogenetically so far apart that a gene probe for one will not hybridise with the other as there is only 65% homology. On the other hand, TEM-1 and TEM-3 have 99% sequence homology,[33] but TEM-3 has the distinction that it can hydrolyse, and confer resistance to, ceftazidime and cefotaxime. This difference has immense clinical significance. Gene probing with a TEM-1 gene probe cannot distinguish it from the TEM-1 gene.

In the clinical environment, change in enzyme structure is a relatively slow occurrence and happens in small stages. Therefore, a scheme distinguishing enzymes or genes must be very sensitive. This problem was ameliorated by the development of iso-electric focusing by Matthew and colleagues.[34] This technique, relying on the characteristic iso-electric point (pI) of enzymes, can, at its highest degree of sophistication, detect single amino acid differences in certain parts of the molecule. An equally sensitive technique, still much in its infancy, is the use of oligonucleotides designed to identify one nucleotide change. However, its success relies on the correct choice of probes. Iso-electric focusing has been the technique universally used and it has been combined with substrate profiles for finer distinction. At first, these compared relative rates at fixed substrate concentrations but, more recently compared relative V_{max} values or relative rates of efficiency (V_{max}/K_m).

Several surveys performed in Europe during the 1980s, employing these techniques,[35–37] demonstrated that the TEM-1 β-lactamase was by far the most prevalent plasmid-encoded enzyme, responsible for up to 80% of the ampicillin resistance mediated by plasmids in gram-negative rods. The next most prevalent enzyme was TEM-2, which varies by one amino acid change from glutamine to lysine at position 37 (on the Sutcliffe numbering scheme). This change produces no apparent alteration in biochemical properties or resistance phenotype. Thus, we can assume that, as far as β-lactam substrates are concerned, these enzymes are identical, and that their relative distributions of about 8:1 come from the relative success of the transposons on which the genes are carried. An equally prevalent enzyme, SHV-1, though phylogenetically distinct, again has very similar properties. The proportion of gram-negative bacteria resistant to ampicillin can vary in the developed world but is usually quite high. A survey in Scotland showed that the proportion was 45% isolates from Edinburgh, but was 70% in similar strains from Glasgow. India has the highest incidence of plasmid-mediated resistance. In 1984, 81% of all Enterobacteriaceae were ampicillin-resistant including 77% of strains *E. coli*.[38] In this study, there was a wide distribution of plasmids of many different types. An epidemic of one plasmid type was not being observed and the proportions of individual β-lactamases was significant. Again, the TEM-1 enzyme was most prevalent, found in >70% of strains. Surprisingly, OXA-1 was more common than TEM-2. However, even in an area where resistance was so prevalent, the number of diffent β-lactamases was quite small. Most of the plasmid-mediated β-lactamases capable of hydrolysing just ampicillin and first-generation cephalosporins have been found on very few occasions.

About 30 of these plasmid-encoded β-lactamases capable

of hydrolysing penicillins and early cephalosporins have been described; however, only five or so are found with any regularity.[39]

The prevalance of the TEM-1 β-lactamase has forced pharmaceutical companies to seek alternative strategies. This has largely been achieved in two ways: (i) the concurrent use of β-lactamase inhibitors; (ii) the development of drugs which are resistant to β-lactamase attack. β-lactam/β-lactamase inhibitors have proved remarkably successful. There are few reports of plasmid-mediated resistance and these have largely been confined to reports of hyperproduction of TEM β-lactamases.

Ceftazidime and cefotaxime were considered, at their launch, to be as close to perfection in the design of β-lactam drugs as was possible to achieve. In one area, it was virtually guaranteed that they would not fail—there would never be plasmid-mediated resistance. In 1982, a change took place which destroyed this guarantee. It was so small that it was missed at the time. A *Klebsiella oxytoca* strain, originally isolated from the blood and CSF of a baby in a neonatal intensive care unit in Liverpool, was gentamicin-resistant but ceftazidime-sensitive and it produced the TEM-1 β-lactamase. A subsequent isolate of *K. oxytoca* from this unit showed that it had now become ceftazidime-resistant. In a series of experiments performed 6 years afterwards, the ceftazidime resistance was found to be carried on a 141-kb auto-transferable plasmid. Biochemical analysis showed that the enzyme hydrolysed ceftazidime at a low, but significant, efficiency. The equivalent enzyme could be obtained by a single-step mutation from the TEM-1 β-lactamase and the Liverpool enzyme was designated TEM--E2.[40] Most of these enzymes have been found in continental Europe. In 1983, three strains of *K. pneumoniae* and one of *Serratia marcescens* were isolated which conferred resistance to cefotaxime. The β-lactamase was plasmid-mediated and a modification of the ubiquitous klebsiella enzyme, SHV-1.[41] A glycine residue at position 236 had changed to serine. There followed an explosion of reports of new, plasmid-mediated extended-spectrum β-lactamases.

There are now nearly as many plasmid-mediated extended-spectrum β-lactamases as there are plasmid-mediated β-lactamases capable of hydrolysing merely ampicillin or early cephalosporins. Almost all of them are derivatives of TEM-1/2 or SHV-1 β-lactamases. The maximum number of mutations is three amino acids from TEM-1, TEM-2 or SHV-1 β-lactamases.

It is difficult to assess whether these enzymes are clinically of great significance. Certainly, there have been outbreaks of resistance caused by these β-lactamases. The best documented was the epidemic of TEM-3 in *K. pneumoniae* strains isolated in French hospitals. This enzyme was found in nearly 500 strains.[42] Recently, there has been an equivalent epidemic of bacteria containing TEM-like enzymes in the USA.[43] Generally, the emergence of these β-lactamases has been sporadic and localised, the vast majority of enzymes being found on very few occasions. This may reflect different localised usages of extended spectrum β-lactam agents, in particular those related to ceftazidime and cefotaxime.

Payne and Amyes[44] have grouped these β-lactamases according to their hydrolysis properties (table II), in a similar manner to the Richmond and Sykes Scheme.[26]

In *Group 1*, there are enzymes which hydrolyse both ceftazidime and cefotaxime poorly. In most cases, they hydrolyse cefotaxime with slightly greater efficiency than ceftazidime—this is paradoxical as they confer greater resistance to ceftazidime, which probably results from the superior penetration of cefotaxime.

Group 2 consists of those enzymes capable of hydrolysing ceftazidime more efficiently than cefotaxime and, generally, confer much higher levels of resistance to this drug. Some would not produce clinically significant resistance to cefotaxime as the level of resistance is insufficient.

Group 3 is the largest group and comprises those enzymes that hydrolyse cefotaxime more efficiently than ceftazidime. However, probably for reasons similar to those with the Group 1 enzyme, resistance to cefotaxime is rarely higher than ceftazidime. We have divided this group into (a) those derived from TEM enzymes, (b) all the SHV-derived extended-spectrum β-lactamases, and (c) two enzymes—one from Japan and one from India—of unknown origins.

All these enzymes confer little, if any, significant resistance to the combination of a β-lactam agent and β-lactamase inhibitor, particularly clavulanic acid. Therefore, such combinations should remain effective. However, a new plasmid-mediated β-lactamase which confers resistance, not only to extended-spectrum β-lactam agents but also to β-lactam/β-lactamase inhibitor combinations, has recently been identified. This enzyme, called BIL-1, appeared in a clinical isolate of *E. coli* in Pakistan.[45] The pI of this enzyme was extraordinarily high at pI 8·8 and thus it could have been confused with the induced β-lactamase of *E. coli*, which is believed to account for the CEP-1 enzyme.[46] The CEP-1 β-lactamase has a pI of 8·2 and was shown to be quite distinct from BIL-1. On the other hand, the BIL-1 β-lactamese does have a very similar pI to the *Enterobacter*

Table II. Plasmid-mediated extended-spectrum β-lactamases

Group 1	Group 2	Group 3	Group 4	Unassigned
TEM-E1	TEM-E3	*TEM-derived*	BIL-1	FUR
TEM-E2	TEM-6	TEM-3		MJ-1
TEM-E4	TEM-9	TEM-4		MJ-2
TEM-7	TEM-10	TEM-5		Unamed 1
CAZ-3	CAZ-7	CAZ-2		Unamed 2
CAZ-lo	CAZ-hi	CAZ-6		
		SHV-derived		
		SHV-2		
		SHV-3		
		SHV-4		
		SHV-5		
		Unknown		
		FEC-1		
		DJP-1		

Group 1, poor hydrolysis of cefotaxime and ceftazidime.
Group 2, better hydrolysis of ceftazidime than cefotaxime.
Group 3, better hydrolysis of cefotaxime than ceftazidime.
Group 4, confers resistance to all cephalosporins and clavulanic acid.

cloacae type A chromosomal β-lactamase. In any case, this enzyme appears to result from a chromosomal gene, encoding a Bush class 1 β-lactamase, migrating into a plasmid vector.

The BIL-1 β-lactamase has virtually no hydrolytic activity against cefotaxime, ceftazidime, cefuroxime or even ampicillin. It does possess activity against nitrocephin and cephaloridine. It has approximately the same relative efficiency of hydrolysis against these two substrates as the ubiquitous TEM-1. The inhibition properties of BIL-1 are much more interesting. It is readily inhibited, and, therefore, binds ampicillin cefuroxime and ceftazidime. It is 4000 times less sensitive to inhibition by clavulanic acid.

There are five enzymes that do not fit into the classification scheme, mainly because there are insufficient data. It is not yet known from where many of these enzymes are derived, and only one has been found on more than two occasions. New enzymes are being discovered constantly and will be entered into this scheme. It is, of course, vital to know the biochemical relationships, because this probably reflects the selective pressures. However, with most resistance mechanisms, classification often takes account of similarities in nucleotide or amino-acid sequences. This requires full gene sequencing to identify the relationships and will be of particular interest with the SHV- and TEM-derived enzymes.

With the SHV-1 enzymes, the relationships are well-defined and are shown in fig. 2. Each box represents a single amino-acid mutation. Therefore, it is easy to see how SHV-1 led to SHV-2 and that SHV-4 was derived either through SHV-3 or SHV-5. All the intervening enzymes have been found in the clinical environment.

The relationships between the TEM-enzymes are much more complicated (fig. 3). Again, each box represents a single amino-acid mutation. It can probably be assumed that the mutation between TEM-1 and TEM-2 occurred before the later enzymes emerged and that the change at amino-acid position 37 does not occur with the extended-spectrum β-lactamases. Therefore, all those mutations in the top half arose from TEM-1 and those in the bottom half from TEM-2. Unlike the SHV enzymes, most of the intermediates have not yet been identified. The laboratory-selected β-lactamase, TEM-101,[47] a mutation of TEM-1, may well be the TEM-E2 we found in the Liverpool *K. oxytoca* isolate;[40] but whether TEM-9, for instance, is derived from intermediate A or D is not known.

Plasmid-mediated β-lacatamases are a vast array of enzymes; there are nearly 60 distinct enzymes. It is almost certain that many more will be identified. The immediate potential clinical threat comes from the extended-spectrum β-lactamases; however, we must wait to see which, if any, of these enzymes will emerge as the successor to TEM-1.

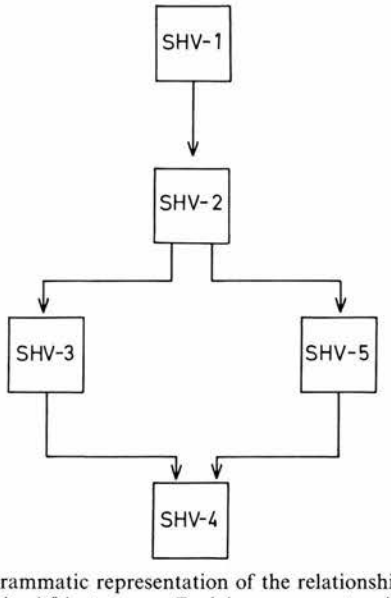

Fig. 2. Diagrammatic representation of the relationships between the SHV-derived β-lactamases. Each box represents a single amino-acid change.

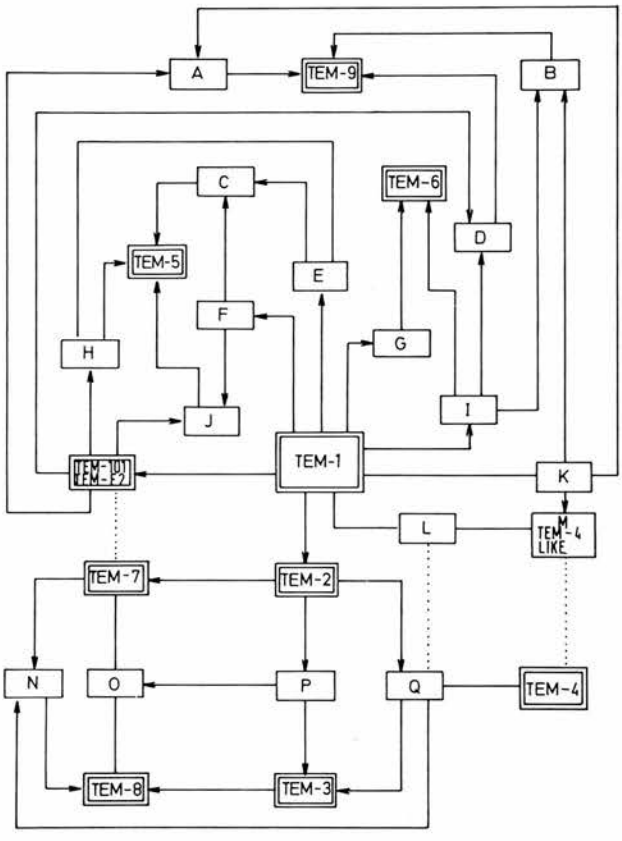

Fig. 3. Diagrammatic representation of the relationships between the TEM-derived β-lactamases. Each box represents a single amino-acid change.

Resistance to the 4-quinolones

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The 4-quinolones are chemically synthesised compounds whose principal target is the enzyme DNA gyrase (E.C. number 5.99.1.3), the only bacterial enzyme capable of introducing negative supercoils into DNA.[48] DNA supercoiling plays an important role in bacterial metabolism—it compacts the chromosome and is involved in the regulation of gene transcription[49] as well as the bacterial response to the environment.[50] The enzyme is a tetramer consisting of two A and two B subunits.[48] The A subunit cuts both strands of DNA simultaneously at intervals four base pairs apart and holds the strands apart but covalently bound to the enzymes. The B subunit, using ATP for energy, then introduces a negative supercoil into the DNA and the A strand then reseals the two strands.

The first 4-quinolone, nalidixic acid, was synthesised almost 30 years ago. Its antimicrobial spectrum is limited to the Enterobacteriaceae; it is useful for the treatment of urinary tract and enteric infections. The antimicrobial spectrum of the 4-quinolones was extended by the discovery that the addition of a piperazine at C6 and fluorine at C7 to the common 4-oxo-1,4-dihydroquinolone skeleton caused a 1000-fold improvement in antimicrobial activity. Bacterial species intrinsically resistant to nalidixic acid are susceptible

to these new fluoroquinolones and, in the 1980s, compounds such as pefloxacin, norfloxacin, ciprofloxacin, enoxacin and ofloxacin, active against a wide range of bacterial species, became available for clinical use.

The increasing therapeutic potential of these antimicrobial agents has caused considerably interest in the mechanisms by which bacterial resistance to the 4-quinolones is mediated. Bacterial resistance to antimicrobials can be mediated either via a plasmid or a chromosomal mutation.

Plasmids often carry several genes conferring resistance to several antimicrobial agents, therefore, in clinically important bacteria, tend to be the main reservoir of resistance to most antimicrobial agents. This is not the case with the 4-quinolones as, unlike other commonly used antimicrobial agents, plasmid-mediated resistance to the 4-quinolones has yet to be identified in clinical isolates. It should be noted that plasmids have been associated with nalidixic acid resistance in shigellas isolated from the Indian subcontinent, but they did confer resistance to the 4-quinolones.[51,52] Although plasmid-mediated 4-quinolone resistance has yet to be found clinically, a potential mechanism of resistance, which may involve drug accumulation, has recently been reported.

The *norA* gene identified in *Staphylococcus aureus* codes for a 50-Kda protein which appears to be membrane associated and confers high-level resistance to hydrophilic fluoroquinolones.[53,54] This gene has been cloned into plasmid pBR322.[53,54] When a quinolone-susceptible strain, *Escherichia coli* HB101, was transformed with the recombinant plasmid, it developed resistance to the 4-quinolones. The MICs of hydrophilic drugs such as enoxacin, norfloxacin, ciprofloxacin and ofloxacin increased 8 to 64 fold whereas only a two-fold increase was observed for the hydrophobic 4-quinolones nalidixic acid and sparfloxacin.[53,54] Kaatz *et al.* have also recently identified a mutation similar to *norA* in an isolate of *S. aureus* which conferred resistance on an *E. coli* recipient strain when it was cloned on to a plasmid (unpublished results). Thus, a mechanism by which a plasmid could confer resistance to the 4-quinolones has been identified although it has yet to be found in a clinical isolate.

The probability of plasmid-mediated 4-quinolone resistance arising via the two other two mechanisms that have been identified for other classes of antibacterial agents seems low. A drug destruction mechanism appears unlikely as the 4-quinolones are synthetic agents. A 4-quinolone resistant DNA gyrase coded by a plasmid would also seem an unlikely candidate because genetic studies have shown that quinolone sensitivity is dominant over quinolone resistance in gyrase genes.[55] Indeed, transformation of quinolone resistant strains with plasmids carrying quinolone susceptible gyrase genes have been used to identify alterations in DNA gyrase as the cause of resistance to the 4-quinolones in gram-negative bacteria.[55–57]

As plasmid-mediated quinolone resistance has yet to be identified in a clinical strain, chromosomal mutations are the only mechanism by which bacteria are able to develop resistance to the 4-quinolones during therapy. Chromosomally-mediated resistance to the 4-quinolones can occur by one of two mechanisms—either an alteration in the target enzyme, DNA gyrase, or a mutation that reduces drug accumulation.

In most species investigated so far, high-level resistance to all 4-quinolones appears to be conferred by mutations in the *gyrA* gene which codes for the A subunit of DNA gyrase. *GyrA* mutations confer high-level cross-resistance to all 4-quinolones but do not seem to be associated with resistance to other, unrelated antibacterial agents. Such mutations have been identified in *E. coli* (cfxA, gyrA, nalA, nfxA),[55,58,59] *Pseudomonas aeruginosa* (nalA, cfxA),[60–62] *Haemophilus influenzae*,[63] *Citrobacter freundii*[64] and *Serratia marcescens*.[65]

The *gyrA* genes of *E. coli*, *Bacillus subtilis*, *S. aureus* and *Klebsiella pneumoniae* have been sequenced revealing close homology between the gyrases of different species.[66–68] Nucleotide sequence analysis performed on 10 spontaneous quinolone-resistant *gyrA* mutants of *E. coli* KL16[69] showed that quinolone resistance appeared to be conferred by point mutations within a small region between amino acids 67 and 106 near the N terminus of the A subunit. Six of the 10 mutants possessed a substitution at amino acid 83, a serine. This mutation has also been identified in a clinical isolate of *E. coli* that developed resistance during enoxacin therapy.[70] In *S. aureus*, this serine is located one amino acid further on at position 84. There is also a serine at amino acid 85 (aa 84 is alanine in *E. coli*) and substitution of either amino acid was found to be the cause of quinolone resistance in clinical isolates of *S. aureus*.[71] Furthermore, high level ciprofloxacin resistance in *S. aureus* (MIC > mg/L) was found to be associated with substitution of both serines.[71] It is interesting to note that all of the mutations conferring quinolone resistance are situated close to the tyrosine at amino acid 122 of the A subunit that is covalently bound to DNA when the enzyme breaks the phosphodiester bonds of DNA.[48] Although little is known about the quinolone-gyrase-DNA complex, it would seem that the alterations in amino acid residues result in disruption of key interactions of the complex.

Mutations in the B subunit of DNA gyrase coded by the *gyrB* gene have been shown to cause 4-quinolone resistance in *E. coli* and *P. aeruginosa*.[55,56] However, unlike *gyrA* mutations, these do not always cause cross-resistance to all 4-quinolones. The *nal31* mutation in *E. coli*[72] confers resistance to the 4-quinolones lacking a C7 piperazine while rendering bacteria hypersensitive to 4-quinolones possessing this substituent. This phenomenon results from the mutation increasing the negative charge of the B subunit, increasing its attraction for the positively charged piperazine group.[73]

The 4-quinolones must penetrate bacteria to gain access to their target DNA gyrase. Therefore, a reduction in the ability of the 4-quinilones to enter bacteria results in decreased bacterial susceptibility. The 4-quinolones penetrate bacteria by diffusion through porins in the outer membrane of gram-negative bacteria. The hydrophobic 4-quinolones such as nalidixic acid are also able to penetrate the bacterium through the phospholipid bilayer.[73,74] Active transport may also play a role in bacterial accumulation of the 4-quinolones as endogenous active efflux of norfloxacin has been demonstrated at the inner membrane of *E. coli* and *P. aeruginosa*.[75–76]

In gram-negative bacteria, mutations affecting 4-quinolone permeability have been identified in *E. coli* (*nalB, nfxB, norB, cfxB*), *Salmonella*, *Pseudomonas* (*nalB, cfxB* and *nfxB, qr1, qr2*), *Klebsiella* and *Serratia* spp. and have been reviewed recently.[52,77] Unlike alterations in DNA gyrase, which confer only cross-resistance to other 4-quinolones, resistance caused by the reduction in 4-quinolone accumulation can be associated with decreased sensitivity to unrelated antibacterial agents. Mutations in *E. coli* which confer resistance to the 4-quinolones by an impermeability mechanism are associated with alterations in outer-membrane porin F (ompF). Both inactivation of the *ompF* gene and mutations in regulatory genes controlling expression of *ompF* at a post-transcriptional level can cause 4-quinolone resistance; e.g., *cfxB* appears to be an allele of the *marA* gene which confers resistance to tetracyclines and chloramphenicol as well as to the 4-quinolones.[78].

In *P. aeruginosa*, resistance to the 4-quinolones resulting from reduced drug accumulation has been associated with alterations in a wide range of outer-membrane proteins.[52] Resistance to the 4-quinolones can be associated with cross-resistance to other antibacterial agents; e.g., the *Qr1* and *Qr2* mutations associated with a reduction in *ompG* and an

antigenically related 40-Kda outer-membrane protein conferred resistance to some β-lactam agents, chloramphenicol and tetracycline, as well as to the 4-quinolones.[76] However, this is not always the case as, for example, unstable low-level resistance to the 4-quinolones in this species associated with the alteration or loss of a 31–32-Kda outer-membrane protein did not confer cross-resistance to antibacterials unrelated to the 4-quinolones.[79]

Fukuda *et al.*[80] have recently identified a new resistance mechanism in *P. aeruginosa* strain PAO associated with reduced drug permeability. The *nfxC* mutation mapped near *catA* (46 min) on the PAO chromosome and caused a 10- to 32-fold decrease in susceptibility to ciprofloxacin, ofloxacin, norfloxacin and fleroxacin. It was associated with increased expression of a 50-Kda protein and decreased expression of a 46-Kda protein. The *nfxC* mutation was associated with cross-resistance to imipenem and chloramphenicol but rendered bacteria highly susceptible to other extended spectrum cephalosporins and to aminoglycosides. This mutation was thought to be clinically significant as quinolone-resistant *P. aeruginosa* that are resistant to imipenem but susceptible to other β-lactam agents have been isolated from patients.[80]

In *E. coli* and *P. aeruginosa*, resistance to the 4-quinolones has been associated with alterations in the expression of lipopolysaccharides.[52,77] Hence, resistance manifested by reduced drug accumulation does not result solely from changes in the outer-membrane proteins. The discovery of an active efflux system for the 4-quinolones suggests that another class of drug mutations, which confer resistance by reducing 4-quinolone accumulation, may exist, although they have yet to be identified. This might be similar to tetracycline resistance, in which the drug efflux system works with enhanced efficiency. Finally, much less information exists about resistance to the 4-quinolones resulting from reduced drug accumulation in gram-positive bacteria. The best characterised mutation is the *norA* mutation in *S. aureus* discussed previously.[54,55]

The development of resistance to the modern fluorinated 4-quinolones during therapy has been shown to have occurred in a wide range of bacterial species.[52,77] These clinical reports indicate that it is possible for most bacterial species to develop resistance to the 4-quinolones during therapy. However, extrapolation of the frequency at which resistance to the fluoroquinolones is likely to develop clinically from these isolated reports is not possible. The monitoring of bacterial susceptibility to the 4-quinolones once they have been introduced serves to provide such epidemiological information. Several large scale studies have monitored the susceptibility of bacterial pathogens to the 4-quinolones since they have become available and, generally, very little resistance to the modern 4-quinolones has been observed.[52,81,82]

Kresken and Wiedemann[81] monitored nalidixic acid resistance in the Federal Republic of Germany, Austria and Switzerland from 1975 to 1986, with particular emphasis on the period between 1983 and 1986 when ofloxacin and norfloxacin became available in these countries. Despite a marked increase in the use of the 4-quinolones during this period, resistance in Enterobacteriaceae, *S. aureus* or *Enterococcus faecalis* did not increase. *P. aeruginosa* was an exception; between 1983 and 1986, the percentage of 4-quinolone-resistant strains increased from 3% to 10%. Another survey performed in the Federal Republic of Germany by Grimm,[82] testing over 100 000 bacterial strains isolated from both hospitals and general practice between 1986 and 1987, found similar results to those of Kresken and Wiedemann[81] for Enterobacteriaceae. However, Grimm[82] report a significant increase in the incidence of 4-quinolone resistance in staphylococci, particularly amongst multi-resistant *S. epidermidis*. There have also been other reports of high incidence of ciprofloxacin resistance in methicillin-resistant *S. aureus* (MRSA) isolated in hospitals.[52,83] This resistance does not necessarily appear to develop during therapy of the MRSA infection may be associated with use of ciprofloxacin for treatment of a pathogen other than MRSA.[52,83] It has been suggested that ciprofloxacin resistance can develop in colonising MRSA which then subsequently spread to other patients in the hospital.[83] Problems may also exist in other bacterial species. The frequency of resistance to the 4-quinolones may be increasing in *Bacteroides fragilis* and *Ser. marcescens*. Surveys in Japan have reported high incidences of quinolone resistance in these species.[66,84]

In conclusion, the clinical incidence of 4-quinolone-resistant bacteria is still relatively rare, although it seems to be occurring with increasing frequency in certain species such as *P. aeruginosa* and staphylococci (particularly multi-resistant organisms). Resistance is not plasmid-mediated at present although a potential mechanism involving reduced accumulation has now been identified. Resistance to the 4-quinolones occurs through chromosomal mutations resulting in an altered target (DNA gyrase) or reduced drug accumulation (particularly associated with alterations in the outer-membrane porins).

MECHANISMS AND EPIDEMIOLOGY OF AMINOGLYCOSIDE RESISTANCE

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Introduction

The aminoglycoside-aminocyclitol antibiotics have remained valuable agents for treating serious infections since their introduction, despite the development of newer broad spectrum and less toxic antibiotics. The emergence of resistance to them has followed their widespread use and, although generally it still remains at a low level for most organisms, is important for the increased mortality, morbidity and expense it causes. This is particularly true for resistant strains having the ability to spread rapidly in institutions causing outbreaks of nosocomial infection.[85]

The uptake of the aminoglycoside antibiotics by the bacterial cell and their mechanisms of action has been shown to be a complex process, as yet incompletely understood. Nevertheless, some discussion of these events is helpful in understanding the different mechanisms of aminoglycoside resistance.

Aminoglycoside uptake and action

The uptake of aminoglycosides by bacterial cells has been most extensively studied with streptomycin and gentamicin. It differs from the uptake of most antibiotics in that it is active (energy-dependent), and has been divided into three phases. The first phase of uptake is the attraction and binding of the antibiotic to the cell outer membrane of gram-negative rods, and is rapid and passive. The second and third phases have been named energy-dependent phases I (EDP-I) and II (EPD-II). The initial passive uptake of aminoglycoside is thought to depend on the electrical potential across the outer membranes. Aminoglycosides are cationic and are, therefore, attracted to anions on the cell surface and across the outer membrane by the internally negative electrical potential, passing through the porin channels of gram-negative cells or water filled interstices of gram-positive cells. In *Pseudomonas aeruginosa*, which has a less permeable outer membrane, a model has been proposed whereby the aminoglycoside binds to the negatively charged

magnesium binding sites on the membrane surface, causing disruption of the membrane barrier and facilitating entry into the cell of aminoglycoside.[86] The extent and rate of ionic uptake depends on the size of the electrical potential gradient across the outer membrane, and a number of inhibitors which reduce ionic binding and diffusion have been described.[87]

The EDP-I appears to be comparatively slow, and is thought to represent the transport of the aminoglycoside across the cytoplasmic membrane to come into contact with the ribosome, perhaps on transporter molecules. It precedes any of the intracellular events leading to cell death and appears to be the rate limiting step in uptake.

The EDP-II occurs as the aminoglycoside binds to specific binding sites on the 30S ribosome and the initiation of protein synthesis is disrupted. This leads to changes in the permeability of the cell membrane and a secondary, rapid escalation in antibiotic uptake, cell disorganisation and, ultimately, cell death.

In aerobic gram-negative bacteria, the kinetics of cell killing has been shown to be biphasic.[88] An initial phase of cell death occurs soon after the aminoglycoside is administered and is thought to be associated with the ionic binding to the cell. It appears to be very rapid and related to the concentration of the antibiotic. A second phase of killing is much slower and independent of drug concentration. Such findings show the importance of obtaining adequate peak aminoglycoside concentrations and have been used as an argument in favour of single, large, once-daily dosage regimens. However, despite this, the slower energy-dependent phases of uptake are the most important phases in the bactericidal action of aminoglycoside antibiotics.[89] Many antibiotics acting on protein synthesis, e.g., chloramphenicol, are bacteriostatic. They do not have the same complex energy-dependent uptake mechanisms as the aminoglycosides, and the interference in protein synthesis by many of these agents is reversible. The uptake of aminoglycosides is irreversible *in vitro* unless the cell membrane is destroyed by an agent such as toluene, and the rapid influx of antibiotic occurring in EDP-II appears to be the important step in the irreversible disruption of protein synthesis conferring cidal activity.[90]

Mechanisms of resistance to aminoglycosides

There are three mechanisms by which resistance of bacteria to aminoglycoside antibiotics may occur—enzymic modification, deficiency of uptake or accumulation, and modification of the ribosomal target site.

Enzymic modification. This is the most common and clinically important cause of acquired aminoglycoside resistance. Enzymes may modify the aminoglycoside by various mechanisms—particularly N-acetylation, O-nucleotidylation or O-phosphorylation. A number of different aminoglycoside modifying enzymes (AME) have been identified and the nomenclature for their description consists of a three letter code according to their mode of action—aminoglycoside acetyltransferase (AAC), aminoglycoside phosphoryl-transferase (APH) and aminoglycoside adenyltransferase or nucleotidylase (AAD or ANT). The site of action of the enzyme on the aminoglycoside molecule is denoted by a number in parenthesis, e.g., AAC(2″) represents acetylation of the 2″-amino group. Further characterisation may be given by the addition of roman numerals to differentiate enzymes of similar actions. Thus, potentially, an aminoglycoside may be modified at more than one site and, therefore, be a substrate for more than one enzyme. Similarly, an enzyme may have several different substrates.

Most genes coding for AMEs are plasmid specified, and many are known to be carried on transposons. Plasmid transfer may occur by conjugation, transduction or transformation and a single plasmid may carry more than one AME gene. More rarely, the enzyme is chromosomally specified, and this has been described in *Serratia* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Staphylococcus aureus* and some streptococci.

In contrast to some other antibiotic-modifying enzymes, e.g., the β-lactamases, AMEs appear to be virtually completely intracellular. Very little enzyme or modified aminoglycoside can be found outside the cell; therefore, extracellular antibiotic is still active. Although the exact location of the AME within the cell is not known, it is thought to be closely associated with the cytoplasmic membrane.[91] Modification of the aminoglycoside substrate at this site could confer resistance by preventing binding to the specific ribosomal binding sites or by interfering with aminoglycoside accumulation. As has been discussed, these processes are closely related.

Enzymes are thought to be constitutively expressed. However, the amount of enzyme produced and the rate of substrate action may be variable. It has been postulated that within each bacterial cell there is competition between the rate of aminoglycoside modification and the rate of transport of aminoglycoside across the cytoplasmic membrane during EDP-I; this being the rate limiting step.[91] If uptake exceeds modification the cell may be killed even though it possesses a mechanism of resistance. With exposure to aminoglycoside, resistant bacterial cells with a higher rate of substrate modification may emerge, perhaps due to selection of bacteria with higher gene copy numbers and hence sufficient enzyme so that modification exceeds aminoglycoside accumulation. At present it is not thought that true enzyme induction occurs.

Deficiency of uptake or accumulation. Because of the complex nature of aminoglycoside uptake there is great potential for resistance to occur by this mechanism. Mutants with altered cell-membrane structure or cellular metabolism could interfere with the aminoglycoside accumulation described above. It is becoming increasingly recognised that isolates that are resistant to aminoglycosides by these mechanisms exist and may have clinical importance.

Most organisms intrinsically resistant to aminoglycosides are unable to accumulate sufficient antibiotic. Strict anaerobes that respire without a functioning electron-transport chain, for example, are unable to drive the energy-requiring phases of uptake and cannot maintain an adequate negative internal potential for uptake to occur. Likewise, some fermentative bacteria, such as streptococci, may exhibit low level resistance due to incomplete electron-transport systems.[92] In some clinical isolates of *P. aeruginosa* showing resistance, an altered lipopolysaccharide structure in the cell membrane has been described;[92] similar findings have been reported with other organisms, particularly *Klebsiella pneumoniae*. Such resistance, as described in *P. aeruginosa*, is chromosomally specified and non-transferable. It is stable *in vitro* and independent of exposure to aminoglycoside. In general, resistance of this type affects a wide range of aminoglycosides and is of low level. High concentrations of aminoglycoside may overcome the permeability barrier and render the organism sensitive. This is in contrast to enzymic modification, which tends to affect specific aminoglycosides and usually produces high-level resistance.

Recently, there has been considerable interest in adaptive resistance as a result of exposure to low levels of aminoglycosides. This has been described both *in vitro* and *in vivo*.[93] Exposure to one dose of aminoglycoside may produce resistant variants of an organism that have altered metabolism and, consequently, impaired aminoglycoside accumulation. This has been shown to occur most readily with *P. aeruginosa*, but also with *Escherichia coli* and *S. aureus*, and, as with the impermeability resistance described above, affects a wide range of aminoglycosides and is of low level.

However, such isolates quickly revert to susceptibility when exposure to the aminoglycoside is removed. This suggests that they are not simply resistant mutants selected by antibiotic exposure. Often, colonies are readily identifiable by their small size, slow growth and atypical morphology,[93] which could also reflect intracellular metabolic changes. Such adaptive resistance may have clinical relevance to cases of treatment failure in conditions requiring prolonged or repeated aminoglycoside therapy, e.g., endocarditis and cystic fibrosis, and could be difficult to detect in the laboratory as isolates may revert to susceptibility at initial isolation.

Modification of ribosomal target site. Aminoglycosides interfere with the initiation of protein synthesis by binding to specific binding sites on the 30S ribosome. As yet, mutations affecting this specific target site do not appear to have great clinical importance. Experimental and clinical isolates showing resistance to streptomycin and spectinomycin by this mechanism have been described, and are thought to arise by single step mutations at the StrA locus. Although mutations near to this site may produce resistance to other aminoglycosides, this has not been seen in clinical isolates.[93]

The epidemiology of aminoglycoside resistance

It is not surprising that resistance to aminoglycosides is becoming increasingly recognised, as these agents have been widely used in hospitals for more than 20 years, not only for treatment but also for prophylaxis. They are also used in veterinary practice and in commercial processes involving animals.[94] Several multicentre study groups have documented the prevalence of aminoglycoside resistance and also the mechanisms of resistance. These include groups in the UK,[95] Europe[96,97] and the USA.[98] Such collaborative studies are essential as the prevalence of aminoglycoside resistance in each individual hospital is low, but they have generally only been able to give a single snapshot of patterns of resistance (the point prevalence). More information can be obtained from studies of the prevalance of resistance over a period time,[99,100] or studies that have been repeated, such as the European Study on Antibiotic Resistance (ESGAR) reports of 1987 and 1990.[96,97] A summary of some of these reports is shown in table III. Variations in the resistance level can be found, not only between countries, but also between laboratories participating in individual studies. One striking difference is the increasing resistance from Northern to Southern Europe (table IV). The USA has resistance levels approaching those of Southern Europe. However, care must be taken when comparing these studies as the isolates examined come from a variety of clinical sources. The majority of studies have looked at consecutive blood-culture isolates, but some have included all clinical isolates or urine isolates. Also, the distribution of organisms varies between studies; those finding a higher incidence of *Providencia*, *Serratia* and *Acinetobacter* spp. and coagulase-negative staphylococci have reported a high level of aminoglycoside resistance. Differences in the geographical pattern of resistance may reflect different patterns of aminoglycoside usage. Similarly, the isolation of organisms more likely to be aminoglycoside-resistant, such as those listed above, may reflect selection secondary to antibiotic exposure.

In all studies, the most common mechanism of aminoglycoside resistance is modification by enzymes. Impermeability resistance accounts for $<10\%$ of resistant isolates and is seen most frequently in non-*aeruginosa Pseudomonas* spp.[95] The distribution of AMEs varies from country to country. In Europe as a whole, the most common AMEs in gram-negative bacilli are ANT(2″), AAC(3)V and ACC(6′)II, and in staphylococci the double-acting AME APH(2″)/AAC(6′) combined and ANT (4′).[96] Combinations of AMEs are common and a wide variety of different enzymes is seen, especially in isolates from Southern Europe where AAC(6′)I, either alone or in combination, is responsible in part for higher resistance levels. In the UK, the most common enzymes are AAC(3) and ANT(2″) in gram-negative bacilli and APH(2″)/AAC(6′) in staphylococci.[95,99] Similar findings have been described from the USA and Australia. This differs from the Far East, where AAC(6″) is most common, and Chile, where $>90\%$ of aminoglycoside-resistant isolates possess ACC(3).[101] Again, these differences may reflect pressure due to antibiotic usage. In Japan, for example, the high usage of kanamycin and dibekacin may have encouraged the selection of AAC(6′), which causes resistance to this group of antibiotics, whereas AAC(3), which confers resistance to gentamicin, sisomycin and netilmicin, is rarely seen. The presence of a single enzyme in resistant Chilean isolates may represent the distribution of a single R-plasmid in a hospital.[101] In studies from other countries, however, there has been a tendency in more recent surveys to find an increasing number of different AMEs, often in combination.[102,103] Although selection pressure from hospital aminoglycoside usage may be the most important factor controlling this, other factors, such as spread of resistance through animals may also be significant.[94]

Table III. Gentamicin resistance (%) described in some recent studies

Study reference	Finland[102]	Sweden[100]	St Thomas's Hospital, UK[99]	Saudi Arabia[103]	Europe[97]		UK[95]		Europe[96]
Number of isolates	633	2027	7264	5001	2578	2463	970	4146	3440
Source of isolates	Blood	Blood	All clinical	All clinical	Blood	Urine	Blood	Urine	Blood
Escherichia coli	0	...	0·6	2	3	3	1·6	0·6	4
Klebsiella spp.	5·5	...	3	9	} 14	} 15	1	8·5	} 10
Citrobacter spp.	...	...	0·5	8			0	30	
Enterobacter spp.	...	...	3	15			6	4	
Serratia spp.	...	...	0	21	30	54	6	28	28
Providencia spp.	...	...	73	53	...	...	...	79	...
Acinetobacter spp.	...	...	13	37	56	50	...	...	65
Pseudomonas spp.	50	...	3	27	28	26	6	6	33
Total all gram-negative bacilli	...	1	5	...	12	12	3·3	2·4	12
Staphylococcus aureus	0·7	1	3	...	12	20	2	5	12
Coagulase-negative staphylococci	20	20	...	...	26	28	29	16	53

Table IV. Aminoglycoside resistance from European blood-culture isolates

Region	Percentage of gram-negative isolates resistant to				Percentage of gram-positive isolates resistant to			
	Gent	Tob	Net	Ami	Gent	Tob	Net	Ami
Northern Europe	3	2	4	3	15	18	6	10
Central Europe	8	7	8	7	32	34	19	28
Southern Europe	24	22	23	15	30	31	17	17

Northern Europe: Sweden Finland, Denmark, Great Britain; Central Europe: Belgium, Netherlands, West Germany, Austria, Switzerland; Southern Europe: France, Spain, Portugal, Italy, Greece.
Gent, gentamicin; Tob, tobramycin; Net, netilmicin; Ami, amikacin.

Methods for identification of resistance mechanism

Some idea as to the mechanism of resistance of an aminoglycoside-resistant isolate can be obtained from the resistance pattern, level of resistance, colonial morphology, and growth, as described above. Most isolates will be resistant by enzymic modification. Identification of the AME is rarely necessary clinically, but may be useful for epidemiology or research. Identification may be presumed by testing susceptibility to the aminoglycoside substrates for the enzyme, and simple methods have been described based upon either MICs[101] or inhibition zone diameters.[104] It is likely that results of these tests will become increasingly difficult to interpret as the frequency of combinations of enzymes increases. Furthermore, some of these methods use, ideally, aminoglycosides not in clinical use and therefore difficult to obtain.

Most other methods for enzyme identification are unlikely to be used by clinical laboratories. Radiolabelled co-factors have been widely used to measure the binding of modified aminoglycoside to cellulose phosphate paper but can give confusing results with some enzyme combinations. High performance liquid chromatography of the reaction products may also be used; this has the advantage of specifically identifying reaction products. Most recently, techniques have concentrated on the genes coding for AMEs. Specific probes have been developed for detecting AME genes, and the polymerase chain reaction can further facilitate this. As techniques improve, these methods will become more sensitive and may prove to be very useful for identifying combinations of AMEs.

GLYCOPEPTIDE RESISTANCE IN GRAM-POSITIVE BACTERIA

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Introduction

Some of the most used antibiotics in clinical medicine are those that affect the bacterial cell wall which is composed of a variety of macromolecules, some of them found uniquely in bacteria. The majority of clinically-important bacteria contain peptidoglycan, which confers rigidity on the bacterium and is the main molecular determinant of cell shape. It is a large, polar molecule which is assembled on the outside surface of the bacterium in three main stages from subunits manufactured inside the cell. The subunits are transferred across the membrane, polymerised to form short polysaccharide chains substituted with unusual peptides which are finally cross-linked to the existing wall by the transpeptidation reactions. This series of reactions leading to the formation of cross-linked peptidoglycan affords a plethora of potential targets for antimicrobial agents.[105] The presence of a unique amino-sugar and the un-natural D-isomers of two amino acids ensures that these targets are unique to bacteria and that antibiotics interfering with the biosynthetic pathway are likely to be selectively toxic to bacteria. The last two reactions in the pathway (polymerisation and transpeptidation) occur outside the cytoplasmic membrane and, consequently, antibiotics that interfere with these stages of synthesis do not require a transport system to reach the target site. Glycopeptide antibiotics (vancomycin and teicoplanin) inhibit the first of these two reactions (and would also block transpeptidation) by binding tightly to the unique acyl-D-alanyl-D-alanine arrangement of amino acids in the membrane-bound nascent peptidoglycan.[106]

Mechanism of action of glycopeptide antibiotics

Glycopeptide antibiotics are effective at low concentrations against the majority of gram-positive bacteria, but toxicity problems in the early years of their application precluded their widespread use in spite of their selective action against the peptidoglycan. The introduction of β-lactamase-stable penicillins resulted in a temporary reduction in the clinical use of vancomycin, but the acquisition of alternative mechanisms of resistance to β-lactam antibiotics in particular, and an improvement in the purity of the vancomycin preparations, witnessed a resurgence in the use of glycopeptides, particularly against multiresistant staphylococci that are intrinsically resistant to all β-lactam antibiotics. Glycopeptide antibiotics, including vancomycin and teicoplanin, are large, rigid molecules; they adopt a bracelet-like configuration with a substantial cleft or pocket into which peptides ending in D-alanyl-D-alanine bind firmly, though non-covalently, with almost computerised precision.[107] It is the mechanism of action of this group—interaction with a structural component of the cell wall rather than with a target protein—that makes this class of antibiotics unique and reduces the possibility of large-scale resistance emerging in clinical strains. Definitive studies of the interaction of glycopeptides with the target peptides were elucidated with the use of nuclear magnetic resonance spectroscopy, by mass spectrometry followed by model building, and by 3-dimensional reconstruction of the binding sites.[108] The wall subunit, either attached to its lipid carrier or as part of the growing peptidoglycan chain to which the new subunit will be added, is held firmly by hydrogen bonding to the peptide backbone of the glycopeptide (fig. 4). The space-filling model of the glycopeptide-pentapeptide complex indicates that the sugars in the wall subunit are not immediately adjacent to the glycopeptide molecule, but the peptidoglycan polymerase whose active site must be located close to the sugars is a large molecule in comparison with both the antibiotic and the wall subunit. Presumably, the

presence of the antibiotic on the subunit prevents the enzyme from being located precisely in the correct position so that it can no longer catalyse the transglycosylation reaction that polymerises the peptidoglycan backbone chain.[109] In addition to the inhibition of the peptidoglycan polymerase by steric hindrance (fig. 5), binding of glycopeptides to the D-alanyl-D-alanine terminus of the subunit effectively blocks the vital transpeptidation reaction that links the growing peptidoglycan chain to the mature cell wall. As the result of the inhibition of these two sequential reactions wall, synthesis is frozen.

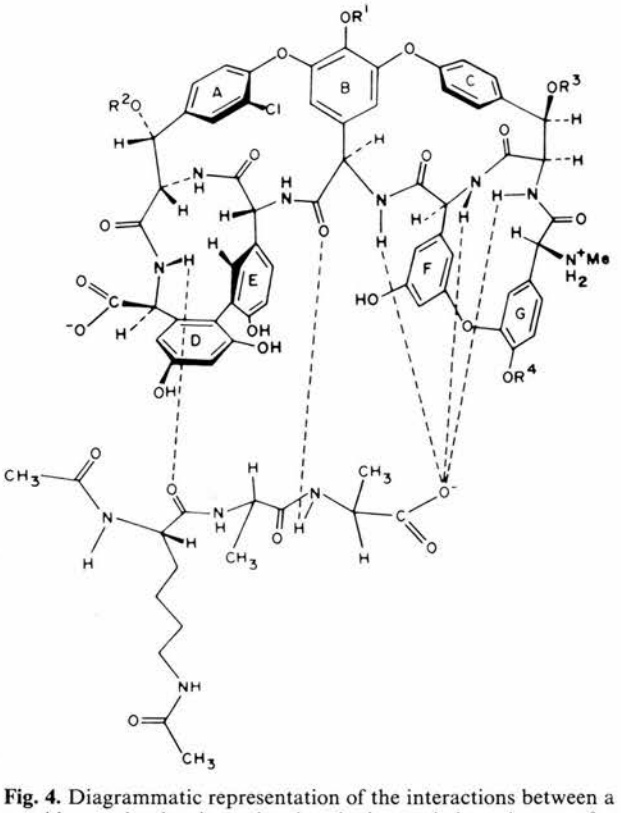

Fig. 4. Diagrammatic representation of the interactions between a peptide terminating in D-alanyl-D-alanine and the aglycone of a glycopeptide: five hydrogen bonds are formed.

Resistance to glycopeptides

The structure of glycopeptides, as well as their unique mechanism of action, is likely to ensure that any resistance mechanism acquired by a bacterium will be unusual compared to conventional mechanisms such as destruction or inactivation of an antibiotic or drastic modification of the target site. Other possible resistance mechanisms include sequestration of the antibiotic molecule by non-specific binding and altered accessibility of the target site. Some bacteria are naturally insensitive to glycopeptides, either because entry through the outer membrane is prevented (gram-negative bacteria) or for an unknown reason (some lactobacilli, leuconostocs, pediococci), though inactivation of the antibiotic molecule has not yet been detected in any instance of resistance.[110]

Sequestration of the available glycopeptide as the result of non-specific binding is also unlikely for various reasons: vast over-production of suitable peptides to complex with the antibiotic molecules would be necessary to raise the MIC of a sensitive strain so dramatically. Such an increase in non-specific binding could occur through secretion of wall precursors into the medium, an enhanced rate of wall turnover involving loss of material into the medium, a substantial increase in the amount of wall peptidoglycan present (and consequent wall thickening) or a decrease in degree of cross-linking in the peptidoglycan with resultant increase in specific binding to non-essential target sites. As yet, none of these scenarios has been detected in investigations of resistant coagulase-negative staphylococci or other resistant (or insensitive) strains;[109] if any of these situations arose there would be a dramatic decrease in the amount of free glycopeptide in the culture medium of resistant strains in comparison with that of their isogenic sensitive strains and no gross increase in glycopeptide binding to resistant

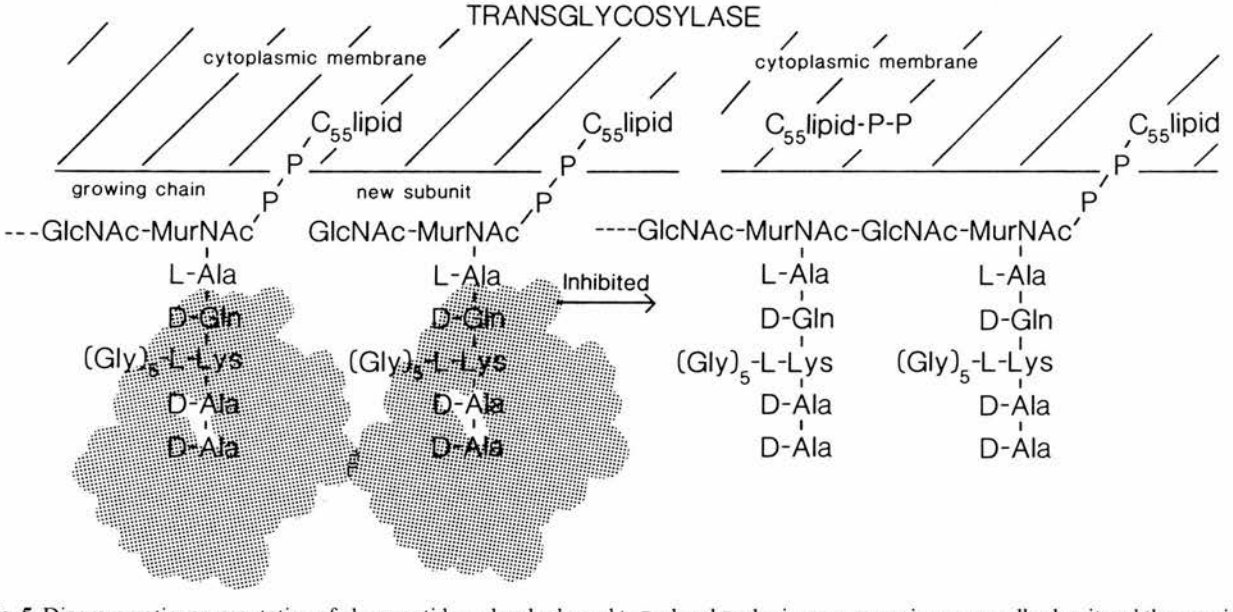

Fig. 5. Diagrammatic representation of glycopeptide molecules bound to D-alanyl-D-alanine sequences in a new wall subunit and the growing nascent glycan chain; these molecules may prevent, by steric hindrance, the transglycosylase reaction that links the nascent chain to the new subunit. The shaded area represents the approximate extent of the glycopeptide. The transglycosylase enzyme is not directly inhibited so resistance cannot result from mutation of the gene encoding the enzyme. Prevention of binding of glycopeptide molecules to the target acyl-D-alanyl-D-alanine grouping could be brought about by inactivation of glycopeptides (not detected); sequestration of antibiotic in the growth medium (not detected); enzymic modification of the target site (difficult for the subsequent essential transpeptidation reaction to occur); or protection of the target site by another molecule (i.e., changing the accessibility to glycopeptides).

bacteria or in complex formation in the culture medium has yet been reported.

Genetic studies

The recently reported resistance in coagulase-negative staphylococci and in enterococci is likely to result either from a change in accessibility of the target or from modification of the target. The resistant enterococci have been studied intensively in several laboratories and at least two types have been identified (table V): high-level resistant strains are resistant to both vancomycin and teicoplanin whereas low-level resistant strains are initially susceptible to teicoplanin and moderately resistant to vancomycin.[111–115]

Resistance is inducible by vancomycin and teicoplanin in the high-level resistant isolates, but only by vancomycin in the low-level resistant strains; however, once resistance to vancomycin is induced, these strains become resistant to teicoplanin as well suggesting that a common factor is involved. The two types of strains differ also with respect to the transferability of resistance to other related or unrelated bacteria. Plasmids encoding resistance have been detected and isolated from some but not all the high-level resistant enterococci[116] and have been transferred by conjugation to glycopeptide-sensitive strains of the same or related species. In one instance, a plasmid has been transferred from its host of *Enterococcus faecium* to a range of other gram-positive bacteria including *Streptococcus sanguis*, *Str. lactis*, *Str. pyogenes* and *Listeria monocytogenes* but not to *Staphylococcus aureus* or *Bacillus subtilis*:[114] if transfer to, and expression of, glycopeptide resistance in *S. aureus* had been achieved, clinical microbiologists would have been very concerned at the prospect of glycopeptide resistance being added to the growing list of antibiotic resistances in multiresistant strains of staphylococci.

Purified plasmid DNA from resistant *E. faecium* has been used to transform *Str. sanguis* Challis to glycopeptide resistance; subsequently a 4-Kb *Eco*R1 fragment containing the gene (*vanA*) encoding resistance was cloned in *Escherichia coli* on a conjugative gram-negative–gram-positive shuttle vector and transferred by conjugation of *Ent. faecalis* and *B. thuringiensis*, which became resistant to glycopeptides.[117]

Transferable resistance in high-level resistant strains is not always associated with plasmid DNA; plasmids were not detected in either the donor or transconjugants in one report of transfer, and in another a strain cured of glycopeptide resistance retained all three plasmids present in the resistant parent.[116] It has been suggested that, in these instances, the resistance gene may have a chromosomal location, possibly in association with a transposon. Such a location has also been postulated for the gene encoding resistance in low-level glycopeptide-resistant strains because resistance is not transferable and no plasmids have been detected. Sequencing of the N-terminus of the VANA protein which is present in high-level resistant strains that have been induced (see below) has enabled the *vanA* gene to be identified and sequenced.[118] The deduced amino-acid sequence corresponded to a protein of calculated Mr $37{\cdot}4 \times 10^3$, similar to that of the protein seen by sodium dodecyl sulphate (SDS)—polyacrylamide gel electrophoresis (39 Kda). The protein encoded by the *vanA* gene appears to be structurally related to the D-alanyl-D-alanine ligases of *E. coli* and *Salmonella typhimurium* (28% and 36% amino acid identity respectively). Furthermore, the *vanA* gene, when introduced into an *E. coli* mutant strain with a thermosensitive D-ala-D-ala ligase, was able to achieve complementation at 42°C.[118]

Biochemical studies

The presence of the *vanA* gene in high-level glycopeptide-resistant strains and its introduction into sensitive strains is associated with the production of a membrane-associated protein of approximately 39 Kda following induction with glycopeptides.[112,119,120] The amounts of the protein in sensitive strains or in uninduced resistant bacteria are very low or non-existent. All high-level resistant enterococcal strains that have been examined contain the *vanA* gene as demonstrated by probing with a 290-bp probe specific for *vanA*, whereas DNA from the low-level resistant strains did not hybridise with this probe.[121] Also, hybridisation was not detected with DNA of the teicoplanin-resistant coagulase-negative staphylococci or the intrinsically resistant leuconostocs, pediococci and lacotobacilli.[121] The presence

Table V. Characteristics of glycopeptide-resistant gram-positive bacteria

Resistance property	Acquired resistance in			Intrinsic resistance* (Lactobacilli, leuconostocs, pediococci)
	enterococci		staphylococci	
	High level	Low-level		
MIC (mg/L)				
vancomycin	≥64	16–32	1–2 (S)	≥1000
teicoplanin	≥16	0·5 (S)	8–32	≥ 250
Transferability	+	−	−	−
Plasmid-mediated	+ (some)	−	−	−
Inducibility by				
vancomycin	+	+	−	Constitutive
teicoplanin	+	−	Constitutive	Constitutive
Mass of resistance protein (Kda)	39	39·5	ND†	ND
Hybridisation to *vanA* probe	+	−	−	−
Reaction with antibody to 39·3-Kda protein	−	+	−	−

(S), sensitive.
ND, not determined.
* This group includes the two glycopeptide-producing strains *Actinomyces orientalis* ATCC 19795 (vancomycin) and *A. teichomyceticus* ATCC 31211.
† Teicoplanin-resistant strains of *S. epidermidis* contain an additional membrane protein (39 Kda) in comparison with a non-isogenic sensitive strain.

of the 39-Kda protein in high-level resistant strains only has been confirmed by immunoblotting with an antibody raised against the purified protein.[122] Low-level resistant strains also synthesise an inducible membrane protein of similar molecular mass (39·5 Kda) which appears to perform a similar function in conferring resistance;[113] although the gene encoding it (designated *vanB* has not yet been sequenced, the N-terminal sequence of the protein was different from that of the 39-Kda protein, and antibodies against the purified protein did not cross-react with the "resistance" protein from high-level resistant strains.[119] A summary of the characteristics of glycopeptide-resistant organisms is given in table V.

Mechanism of resistance—hypotheses

The balance of evidence suggests that resistance in the enterococci results from altered accessibility of glycopeptide molecules to their target sites. A reduction in accessibility could be due either to production of the target cell-wall peptides (acyl-D-ala-D-ala) by another molecule so that glycopeptide molecules are prevented from binding, or to enzymic modification of the target so that glycopeptides no longer recognise the target and cannot bind to it (or bind with much lower affinity).

Modification of target. The product of the *vanA* gene has considerable homology with D-ala-D-ala ligases from gram-negative organisms. The specificity of these enzymes is not absolute and, with appropriate substrate pressure, it is conceivable that a wall precursor could be synthesised in which the terminal D-ala-D-ala was replaced with a different dipeptide.[121] Theoretical considerations render this suggestion unlikely: the introduction of cross-bridges between peptides in the peptidoglycan depends on transpeptidation, which in turn requires the two C-terminal amino acids of the wall precursor to be in the D-configuration. Even if one or both amino acids could be altered to other D-amino acids under normal physiological conditions, it is doubtful if the binding affinity of glycopeptides to the peptide would be changed substantially.

Strong experimental evidence for a different type of modification comes from investigations in which it was shown that wall-membrane fragments of both high- and low-level resistant enterococci prevented the binding of vancomycin and teicoplanin to a soluble pentapeptide terminating in acyl-D-ala-D-ala.[123] In the absence of the wall-membrane fragments, or with an identical preparation from sensitive or uninduced resistant enterococci, binding of vancomycin to the added peptide was not prevented. This is an excellent model system for studying the binding of glycopeptides to their target sites and it was shown that prevention of binding was time dependent; it could be blocked by adding a large excess of the dipeptide D-ala-D-ala (which does not itself bind to glycopeptides), and it was destroyed by heating the wall-membrane preparation—these observations are consistent with the involvement of an enzyme in the protection phenomenon.[123] Furthermore, in these experiments there was a high molar excess of pentapeptide to induced 39-Kda protein which rules out simple binding as a resistance mechanism. It was postulated that enzymic modification may have involved the release of D-alanine from the pentapeptide,[123] though such an activity would prevent subsequent transpeptidation if it occurred *in vivo*. There are two lines of evidence not in accord with this hypothesis: the resistant enterococci are susceptible to some derivatives of vancomycin,[122] suggesting that the binding site for glycopeptides has not been changed substantially, and, more significantly, treatment of the wall-membrane preparation of the resistant enterococci with SDS at 100°C permitted binding of vancomycin to the residual wall whereas no binding occurred to the native preparation.[123] This suggests that no drastic modification of the glycopeptide binding site has occurred.

Protection of target. Two lines of evidence suggest that the additional membrane protein present in resistant strains of enterococci that have been induced with glycopeptides recognises D-ala-D-ala, the binding site of glycopeptides. The VANA protein has considerable homology with D-ala-D-ala ligases[118] (as discussed above) and the prevention of binding of vancomycin to a synthetic pentapeptide terminating in D-ala-D-ala by a wall-membrane preparation containing either the 38-Kda or the 39·5-Kda protein was eliminated by an excess of D-ala-D-ala.[123] However, simple binding of the additional membrane protein to the glycopeptide binding sites is unlikely in view of the large number of these sites (2×10^7 in a staphylococcus), unless the topographical distribution of the protein in the membrane protects the wall precursors as they are extruded through the membrane and until they are fully incorporated into the mature wall.

Glycopeptide resistance in coagulase-negative staphylococci

The degree of resistance to glycopeptides in coagulase-negative staphylococci, particularly *S. epidermidis* and *S. haemolyticus*, is not as great as with the enterococci. Furthermore, these isolates are resistant to teicoplanin but not to vancomycin.[124] This implies that the resistance mechanism will be different from that in the enterococci and could be accounted for by slightly altered accessibility. An additional membrane protein of 39 Kda has been found in resistant isolates of *S. epidermidis* in comparison with sensitive isolates (non-isogenic) but there is no direct evidence that this protein is involved with resistance except by analogy with the enterococci (M. D. O'Hare and P. E. Reynolds, unpublished observations).

Summary

Selective antibiotic pressure due to the increased use of glycopeptide antibiotics for the control of infections caused by gram-positive bacteria has resulted in the emergence of resistance in enterococci and coagulase-negative staphylococci. It is of particular concern that resistance is mediated in some enterococcal strains by self-transferable plasmids with a wide hose range; such a situation may lead to the transfer of this type of glycopeptide resistance to other gram-positive pathogens.[121]

Although glycopeptide antibiotics have been used for 30 years, cases of resistance have been slow to emerge. This is probably due to the unique interaction between a compact and relatively rigid antibiotic molecule and its target, which is, unusually, a substrate for peptidoglycan synthesis rather than an enzyme involved in catalysis. For this reason, any mechanism of resistance is likely to be unusual: diminished accessibility of glycopeptides to their target sites is the most likely possibility in both enterococci and coagulase-negative staphylococci but whether this results from target protection or modification has not yet been demonstrated convincingly.

SURVEILLANCE OF ANTIBIOTIC RESISTANCE IN ENGLAND AND WALES

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Surveillance of antibiotic resistance may be done at both local and national levels. At local level, the compilation of

results of routine diagnostic work for surveillance purposes is readily achieved, particularly if the work of the laboratory is computerised.[125,126] The data derived may be used in the formulation of local prescribed guidelines and policies.[127,128] Special studies and surveys are also undertaken at local level and may involve additional susceptibility testing and investigation of the mechanisms and genetics of particular resistance markers detected. Such studies may relate to local experience with particular pathogens, e.g., enterococci in Nottingham,[129] or cover prolonged periods of study for organisms isolated from particular sites of infection, such as blood, e.g., Phillips and co-workers reported recently on the antibiotic susceptibility of bacteraemia at St Thomas' Hospital over a 20-year period.[127]

For the definition of national trends in antibiotic resistance, cultures isolated in different laboratories may be referred to one centre, where they can be examined for antibiotic susceptibility with standardised methods. Such studies may be specifically organised for this purpose[130,131] or result from the examination of cultures submitted to reference laboratories for typing or other specialised studies.[132,133] The number of different cultures, participating centres, and time periods of study that may be included in specially organised surveys are necessarily restricted by resource and staffing implications. Similarly, data derived from reference laboratory studies may be biased because the criteria for submission of status for reference work are not standardised.

An alternative approach to national surveillance is provided by the referral of results of susceptibility tests (done for diagnostic and therapeutic purposes) from diagnostic laboratories to one or more centres where they are compiled and analysed. Large multi-centre studies spanning several years have been performed in the USA[134] and the UK.[135] The recent UK report by Spencer and co-workers utilised a computer-based reporting scheme called Microbase[136] to collect susceptibility data on nearly 367 000 isolates from 61 medical microbiology laboratories distributed throughout much of the UK.[135]

The remainder of this paper describes an approach to the continuous surveillance of antibiotic resistance which utilises and expands the existing scheme for reporting bacterial isolates from cases of bacteraemia and meningitis to the PHLS Communicable Disease Surveillance Centre (CDSC).

The CDSC operates a voluntary, confidential, laboratory reporting scheme for a wide range of infections in England and Wales. Within the CDSC reporting scheme, laboratories are requested to report significant bacterial isolates from cases of bacteraemia and meningitis. During 1988, a major re-organisation of this reporting scheme was initiated involving the introduction of new computers, software and report forms. This re-organisation suggested to staff within the Division of Hospital Infection (DHI) of the Central Public Health Laboratory (CPHL) an opportunity to undertake continuous surveillance of antimicrobial susceptibility data for bacteraemia and meningitis isolates reported to CDSC. Accordingly, after consultation with colleagues in CDSC, the new Communicable Disease Report (CDR) Form 2 was modified to include a request to the reporting laboratories to list the antimicrobial susceptibility test results obtained from routine diagnostic work on the cultures being reported. This approach has several advantages. Firstly, the data collected relates to organisms judged responsible for significant infection. Secondly, the only additional workload required in the reporting laboratory is to add their own susceptibility test results to the form they are already completing. Thirdly, the majority of all diagnostic microbiology laboratories in England and Wales participate in the reporting scheme giving very wide coverage. Fourthly, the results obtained can be analysed geographically and by clinical and epidemiological features (where these data are available); and finally the surveillance scheme can run continuously over many years providing a unique database providing trends in antibiotic resistance. The new report forms, with the request to include susceptibility test results, were introduced at the beginning of 1989. This paper presents some of the results obtained for bacterial isolates reported during the first half of the 1989.

Reporters were asked to enter antibiotic susceptibility test results as susceptible, intermediate or resistant (using their own definitions), for the bacterial isolates they were reporting. The susceptibility test results were added to the existing CDSC computer database records of individual patient infections (containing details of the source laboratory, and clinical and epidemiological features) by staff of the Division of Hospital Infection. As only those agents to which clinical isolates were routinely tested in the individual laboratories were included in the reports, there was considerable variation in the total number of tests for each antibiotic. Generally, we have selected for inclusion those antibiotics for which a result appears in $\geqslant 40$–50% of reports for a particular species. When positive isolation was reported from both blood and CSF in cases of meningitis, infection was counted only once. Therefore each organism included in these totals relates to an individual patient's infection.

In this preliminary report it is not feasible to present the wealth of data that is being generated by this surveillance system. Therefore, we have chosen for inclusion data relating to four pathogens commonly reported to CDSC as causing bacteraemia or meningitis, or both. Tables VI–IX show the reported susceptibility test results for *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. The number of different laboratories from which the reports were received is also shown. In reports of these four bacterial infections, 84–87% included results of antimicrobial susceptibility tests and 87–92% of individual reporting laboratories included such data in their reports.

For those agents most likely to be used therapeutically for *E. coli* bacteraemia (table VI)—the cephalosporins and aminoglycosides—full susceptibility was reported for >98% of isolates with the exception of cefuroxime, for which full susceptibility was reported for 92·6%. Susceptibility to ampicillin or amoxycillin was reported for 45% and susceptibility to trimethoprim for 80% of the isolates tested.

Amongst the 2366 *S. aureus* bacteraemias reported from 249 laboratories, susceptibility test results were included for 87% (table VII). Apart from the 85% of isolates resistant to penicillin, little antibiotic resistance was reported for the agents tested, with 92% sensitivity to erythromycin, 98% to methicillin, and 97% to gentamicin and fusidic acid.

The pneumococcus was the third most common organism reported. Of these reports, 85% included susceptibility test results. Full susceptibility to penicillin was reported for almost all; with only three of 1701 isolates were reported to be resistant or of intermediate sensitivity to penicillin. Of nearly 1600 isolates for which reports included details of erythromycin susceptibility, 2·5% were reported as resistant or of intermediate susceptibility. Nearly 99% of the 385 isolates for which chloramphenicol susceptibility was reported were recorded as sensitive. This total includes virtually all those from meningitis but relatively few of those from bacteraemia.

Susceptibility test results were received for 86% of 499 cases of bacteraemia due to *Pseudomonas aeruginosa* reported from 184 laboratories (table IX). Susceptibility to gentamicin was noted in 91% of 338 reports, 1·5% were reported to be of intermediate susceptibility and 7·4% resistant to gentamicin. Susceptibility to ciprofloxacin, ceftazidime, piperacillin and azlocillin was reported for 95%, 94%, 95% and 90% of isolates respectively amongst reports that included results for these antibiotics.

The results obtained to date and the continuing support that the surveillance scheme is receiving from microbiologists in England and Wales suggest that this approach to surveillance is likely to be useful and relevent for the foreseeable future. It should be possible to monitor and report trends and shifts in antibiotic susceptibility for a wide range of common pathogens causing bacteraemia and meningitis.

The accuracy of results reported in such a surveillance study could be questioned. However, the results received are those generated during normal laboratory testing for diagnostic and therapeutic purposes. Such tests are subject to both internal and external quality control. Results from the National External Quality Assurance Scheme for microbiology show that, for most organism and antibiotic combinations examined within the scheme, agreement with the designated correct result is achieved on 80–100% of occasions, (Personal Communication, J.J.S. Snell). Thus, it seems reasonable to accept the reported results as valid.

For *E. coli,* the results obtained for gentamacin susceptibility are identical to those obtained in the 3-year survey reported by Spencer[135]—1% resistance—and very similar to the 1·6% gentamicin resistance amongst 371 blood-culture isolates reported from the Bristol survey in 1986.[131] The latter two studies reported 97% and 98% of *E. coli* strains to be sensitive to cefuroxime, compared with the 92·6% reported here. It remains to be seen whether there is any significant trend towards resistance to cefuroxime in isolates

Table VI. Antimicrobial susceptibility of *E. coli* isolates from cases of bacteraemia reported to CDSC/DHI from England and Wales, Jan.–June 1989

Antibiotic	Number (%) tested	Number (%) of strains that were		
		S	I	R
Ampicillin/Amoxycillin	3070 (97·5)	1400 (45·6)	119 (3·9)	1551 (50·5)
Cefotaxime	1554 (49·4)	1542 (99·2)	3 (0·2)	9 (0·6)
Ceftazidime	1046 (33·2)	1036 (99·0)	4 (0·4)	6 (0·6)
Cefuroxime	2189 (69·6)	2027 (92·6)	72 (3·3)	90 (4·1)
Ciprofloxacin	1572 (50)	1568 (99·7)	3 (0·2)	1 (0·1)
Gentamicin	3015 (95·8)	2958 (98·1)	27 (0·9)	80 (1·0)
Trimethoprim	2261 (71·8)	1816 (80·3)	19 (0·8)	426 (18·8)

Total number reported	3586
Number of reporting laboratories	254
Number (%) with one or more sensitivity results	3147 (87·8)
Number (%) of laboratories reporting sensitivity results	237 (93·3)

S, sensitive; I, intermediate sensitivity; R, resistant.

Table VII. Antimicrobial susceptibility of *S. aureus* isolates from cases of bacteraemia reported to CDSC/DHI from England and Wales, Jan.–June 1989

Antibiotic	Number (%) tested	Number (%) of strains that were		
		S	I	R
Penicillin	1995 (96·6)	292 (14·6)	. . .	1703 (85·4)
Erythromycin	1981 (95·9)	1830 (92·4)	4(0·2)	147 (7·4)
Methicillin	1962 (95·0)	1920 (97·9)	. . .	42 (2·1)
Gentamicin	1623 (78·6)	1571 (98·8)	14 (0·9)	38 (2·3)
Fusidic acid	1659 (80·3)	1618 (97·5)	5 (0·3)	36 (2·2)

Total number reported	2366
Number of reporting laboratories	249
Number (%) with one or more sensitivity results	2065 (87·3)
Number (%) of laboratories reporting sensitivity results	237 (92·0)

Table VIII. Antimicrobial susceptibility of pneumococci isolated from cases of bacteraemia and meningitis reported to CDSC/DHI from England and Wales, Jan.–June 1989

Antibiotic	Number (%) tested	Number (%) of strains that were		
		S	I	R
Penicillin	1701 (98·5)	1698 (99·8)	2 (0·1)	1 (0·06)
Tetracycline	756 (43·8)	735 (97·2)	9 (1·2)	12 (1·6)
Erythromycin	1596 (92·4)	1551 (97·2)	4 (0·3)	41 (2·5)
Chloramphenicol	385 (22·3)	380 (98·7)	1 (0·3)	4 (1·0)

Total number reported	2040
Number of reporting laboratories	244
Number (%) with one or more sensitivity results	1728 (84·7)
Number (%) of laboratories reporting sensitivity results	229 (93·6)

Table IX. Antimicrobial susceptibility of *P. Aeuruginosa* isolates from cases of bacteraemia reported to CDSC/DHI from England and Wales, Jan.–June 1989

Antibiotic	Number (%) tested	Number (%) of strains that were		
		S	I	R
Azlocillin	174 (49·6)	156 (89·7)	6 (3·4)	12 (6·9)
Ceftazidime	247 (70·4)	233 (94·3)	3 (1·2)	11 (4·5)
Ciprofloxacin	288 (82·1)	275 (95·5)	7 (2·4)	6 (2·1)
Gentamicin	338 (96·3)	308 (91·1)	5 (1·5)	25 (7·4)
Piperacillin	169 (48·1)	160 (94·7)	3 (1·8)	6 (3·6)

Total number reported	409
Number of reporting laboratories	184
Number (%) with one or more sensitivity results	351 (85·8)
Number (%) of laboratories reporting sensitivity results	160 (87·0)

of *E. coli* from bacteraemia. The presence in these data of a small number of reports of resistance or intermediate sensitivity to cefotaxime and ceftazidime at 0·8% and 1%, respectively, of isolates tested against these antibiotics is suggestive of emerging resistance to these agents. As this survey continues it should be possible to plot any such changes in susceptibility to cephalosporins and, indeed, to other antimicrobial agents over time.

Amongst the isolates of *S. aureus* from bacteraemia, susceptibility to gentamicin and methicillin was reported in 97% and 98% respectively. These results are similar to the 98% sensitivity to gentamicin for 204 blood-cultures isolates of *S. aureus* reported in Bristol[131] and the 98% sensitivity to methicillin reported for Spencer.[135]

Amongst pneumococci, very low levels of resistance to penicillin and chloramphenicol were reported. However, it should be noted that strains resistant to these agents are responsible for significant infections in patients in England and Wales, albeit in very low numbers. Resistance to erythromycin was noted in 2·5% of nearly 1600 reports of isolates from blood or CSF, compared with the 1% of >7000 pneumococci from various clinical sources reported by Spencer.[135] Whether this small difference represents a change in overall susceptibility to erythromycin may become apparent as the survey continues.

The 7·4% incidence of resistance to gentamicin amongst 338 bacteraemia isolates of *P. aeruginosa* is notably higher than the 1% resistance reported for *Pseudomonas* spp.[135] and a little higher than the 4% resistance reported for 23 blood isolates in Bristol.[131] A survey of 1866 isolates of *P. aeruginosa* collected from 24 British hospitals noted resistance in 5·5%.[137]

With a few exceptions the results reported here present a reasonably reassuring picture of continued bacterial susceptibility to a wide range of antibiotics. Comparison of these results with those obtained from culture-based surveys shows reasonable concordance. The continuous surveillance of the antibiotic susceptibility of bacteraemia isolates by the present system, supplemented with occasional culture–based surveys of particular species to define precisely the genetics and mechanisms of resistance, offer a simple and cost effective approach to national surveillance.

Is there any relationship between bacterial resistance and virulence?

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To access the association between virulence and antibiotic resistance, it is essential to examine the interaction from two aspects—firstly, in relation to plasmid encoded resistance and secondly for chromosomally mediated resistance and virulence; the interplay may be demonstrated by the use of appropriate animal models. Many questions on this topic have been raised and a multitude remain unsolved. For an organism to be virulent it must possess some features or factors which may include: (i) the ability to adhere to other cells (*via* pili, fimbriae or adhesins); (ii) the production of an extracellular toxin; or (iii) the ability to penetrate the tissues or cells by virulence factors (once inside the cell the bacteria may multiply and destroy it). Several of these features are exquisitely plasmid mediated, others are only chromosomal.

Many virulence or pathogenicity factors are known to be encoded by plasmids. Some are toxins that cause diarrhoea; some are factors responsible for the adhesion of bacteria to other cells; some are components that allow bacteria to penetrate HeLa cells and then multiply; and some are features that enable salmonellae to persist in various organs. Many of these virulence factors happen to be associated with antibiotic resistance markers carried on plasmids. One of the best known instances of a linkage between resistance and virulence conferred by a plasmid is that of enterotoxin production amongst antibiotic-resistant *Escherichia coli* isolates in the Far East.[138] Toxigenic *E. coli* produce either a heat-labile, antigenic, large-molecular-weight toxin (LT) or a heat-stable, non-antigenic toxin (ST), or both. These *E. coli* strains contain plasmids that carry the genetic information necessary for the production of these toxins. Several conflicting reports suggested that *E. coli* may or may not be able to carry together plasmids conferring enterotoxin production and antibiotic resistance, but Echeverria *et al.* collected 176 enterotoxigenic *E. coli* (ETEC) strains for the Far East, of which 72% were resistant to one or more antibiotic; 44% were resistant to four or more agents. In transfer experiments, 80% of 31 ETEC strains transferred part of their resistance pattern and 35% also transferred their toxigenicity profile. None of the recipients acquired toxigenicity without antibiotic resistance. These workers concluded that ETEC isolated from the Far East in 1978 were frequently resistant to antimicrobial agents, and that genes coding for resistance and enterotoxin production could be transferred together *in vivo*. It was not known if this phenomenon could occur in the human gastrointestinal tract.

This type of association has also been observed by other workers. Wachsmuth *et al.*[139] found that 36% of a multi-resistant *E. coli* population produced ST and that all toxigenic strains were resistant to antibiotics. Smith and Linggood[140] examined ETEC isolates from pigs and found that toxin-conferring plasmids were transmitted with resistance plasmids and, although not linked, the plasmids were transferred together. Because interbacterial transfer of antibiotic resistance occurs more efficiently in animals when

antimicrobial agents are used, caution was advocated against "the widespread use of antibiotics because it might result in an increased incidence of bacteria possessing pathogenic plasmids in the environment of man and domestic animals".

The precise mechanisms of virulence of enteropathogenic *E. coli* (EPEC) are not known. However, work by Laporta *et al.*[141] has shown that EPEC adhere to HeLa cells in two different ways. These are termed localised adherence (LA) and diffuse adherence (DA). The bacteria demonstrating LA stick to localised areas of the cells in which they form distinct microcolonies or clusters, whereas the isolates showing DA adhere to the whole surface of the HeLa cells. The LA characteristic has been shown to be consistent in EPEC serotypes and focal adherence of *E. coli* O111 : H cells to small bowel epithelia and packed aggregates on HEp-2 cells have been described previously by Clausen and Christie.[142]

Laporta *et al.*[142] confirmed that naturally occurring plasmids encoding both LA and antibiotic resistance have been recognised in *E. coli*. These workers examined six strains of EPEC possessing transferable drug resistance; two strains possessed the characteristic of joint factor expression. One strain carried two plasmids, one coding for ampicillin (Ap) resistance and the other for LA, whereas the other strain contained only one plasmid coding for resistance to four antibiotics and LA. Curing by acridine orange of an $ApLA^+$ transconjugant showed that both features were lost simultaneously.

The situation is different in bacteria possessing chromosomal mutations conferring antibiotic resistance. One of the best known examples of this is an alteration in a porin, a protein component (Omp) of the outer membrane of gram-negative bacteria responsible for the movement into the bacterial cells of many chemicals such as nutrients, ions and certain antibiotics. A very complex control mechanism regulates the quantitative production of porins and affects their sensitivity to different families of antibiotics such as β-lactams, chloramphenicol, tetracycline and the quinolones. The porins span the outer membrane as protein trimers that admit small hydrophilic molecules to the cytoplasm. In *E. coli,* Omp F has been shown to be larger than Omp C (1·2 nm compared with 1·1 nm). Thus, the ratio of Omp F. Omp C affects the range of chemicals and antibiotics admitted to the cell. Genes coding for the control of Omp F and Omp C are under the transcriptional regulation of the Omp R gene, which itself is part of the component regulatory system Omp R and env Z. The env Z gene encodes a transmembrane sensory component which acts as a donor for the product of Omp R, a transcriptional regulator. Dorman *et al.*[143] introduced, via transposon-generated mutation, into a mouse-virulent strain of *Salmonella typhimurium*, various Omp changes, which included Omp C, Omp D (another porin present in salmonellae), Omp F and Omp R. Once characterised, the mutated strains were inoculated into BALB/c mice by either the oral or intravenous route. In comparison with the parent strain, Omp C- or Omp F-deficient strains were identical in virulence. The Omp D mutant was slightly less virulent whereas the mutant Omp R failed to kill the mice after oral challenge and showed a markedly reduced intravenous LD50. Furthermore, the Omp R mutants persisted for several weeks in murine tissues, and appeared to protect against subsequent challenge by the parent strain.

Cyclic AMP (cAMP) and the cAMP receptor protein (CRP) are essential for the transcription of many genes and operons involved in catabolite transport and breakdown. The cAMP levels in cells influence various factors related to virulence or pathogenicity, such as synthesis of fimbriae and flagella and manufacture of at least one Omp. Deficiency in cAMP and CRP can also be associated with β-lactam resistance. Curtis and Kelly[144] developed strains of *Salmonella typhimurium* which were unable to synthesise adenyl cyclase and CRP and thus were deficient in cAMP. The parent strain of *S. typhimurium* possessed a virulence plasmid, pStSR100, which enabled it to attach to, invade and persist in Peyer's patches but was defective in traversing to the mesenteric lymph nodes and spleen. With several different genotypic mutant derivatives, studies were performed to assess the following properties; carbohydrate, metabolism, flagellum synthesis, colony size, virulence in BALB/c mice, genetic stability, tissue tropism, persistence of avirulent mutants and immunogenicity. All of the mutant strains were less virulent and induced a high level of protective immunity. Tissue tropism and persistence were not markedly different in the cAMP and CRP mutants. However, the genetic mutations created appeared to be stable. The infective dose required for the mutants was significantly higher than that of the parental strain, whereas the colony size and generation times of the mutant strains were smaller and longer than the wild type. Thus, mutations causing loss of adenyl cyclase or loss of CRP lead to avirulent but immunogenic strains of *S. typhimurium*.

Shigella flexneri causes dysentery in man by penetrating and multiplying inside human colonic epithelial cells. This invasive process has been demonstrated *in vitro* by showing HeLa cell invasion and a subsequent cytopathic effect. The expression of genes located on the chromosome is required for full virulence, but loci encoding for entry into epithelial cells are located on a 220-kb virulence plasmid. Bernadini *et al.*[145] constructed mutants of the Omp B locus which contains the Omp R and env Z genes, which in turn are responsible for regulatory changes in accordance with environmental stimuli. Deletion of the Omp B locus via an allelic exchange yielded a mutant that expressed a low level of entry into cells and a limited capacity to survive intracellularly. Other virulence assays confirmed that the virulence of the mutant was severely impaired.

These three examples of chromosomal mutations which were associated with decreased virulence have shown that genetic changes which are known to be related to antibiotic resistance (although it was not tested in these particular mutants) may have a price in terms of viability, persistence and invasiveness. Two examples follow, in which sensitive clinical isolates of bacteria resistant *in vitro* have been investigated with respect to virulence in a mouse infection model.

Resistance to β-lactam and aminoglycoside antibiotics in *Pseudomonas aeruginosa* may be conferred either by enzymes (plasmid or chromosomal) or by a change to permeability. When aminoglycoside resistance is present it may result from either a complete loss or significant reduction of the O-antigenic subunits of lipopolysaccharide. Bryan *et al.*[146] examined the virulence of a series of mutant and transductant strains of *P. aeruginosa* that expressed β-lactamase and aminoglycoside resistance, in a mouse model of peritonitis and septicaemia (table X). The mechanisms of resistance of these mutant strains included (1) for β-lactam antibiotics, the production of a constitutive β-lactamase, a decrease in permeability and a decreased affinity for penicillin-binding proteins, and (2) for aminoglycoside antibiotics, a decrease in cytochrome and nitrate reductase as the absence or reduction in the subunits of LPS. The results for these various mutant strains in the mouse peritonitis model revealed that bacteria showing mechanisms of resistance to β-lactam antibiotics all retained virulence properties similar to the parent. This is consistent with observations of these isolates that showed no change of morphology, growth rate or production of exotoxin A. However, the aminoglycoside-resistant strains showed a significant decrease in virulence. Three strains with altered cytochrome demonstrated slower growth rates and two mutant strains had "uncapped" LPS. Thus, in contrast to strains resistant to β-lactam agents,

Table X. Strains, properties and features of *P. aeruginosa* strains[146]

Strain no.	Resistance mechanism	MIC (mg/l)		Virulence	LD50	Growth rate
		Piperacillin	Gentamicin			
PA 0503	None	1	1	—	7×10^5	Normal
β-lactam resistant						
PCC 118	Constitutive β-lactamase, permeability decrease to β-lactamase	>120	1	—	$5{\cdot}5 \times 10^5$	Normal
PCC 45	Permeability decrease	4	1	—	6×10^5	Normal
PCC 17	Decreased affinity to PBP	80	1	—	5×10^5	Normal
PCC 2	Decreased affinity to PBP 3	4	1	—	6×10^5	Normal
Aminoglycoside resistant						
PAO 2401	Cytochrome and NO_3 reduction	1	15	↓	$72{\cdot}1 \times 10^6$	↓
PAO 2402	No cytochrome and NO_3 reduced	1	4	↓	$72{\cdot}1 \times 10^6$	↓
PAO 2403	No cytochrome and NO_3 reduced	1	16	↓	$72{\cdot}1 \times 10^6$	↓
PAO 503–18	Absence of LPS subunit	1	4	↓	$72{\cdot}1 \times 10^6$	Normal
PAO 503–16	Marked reduction in LPS subunit	1	4	↓	$72{\cdot}1 \times 10^6$	Normal

those with reduced aminoglycoside sensitivity manifested reduced virulence. Similar mutant strains have been isolated from cystic fibrosis patients, almost all of whom had repeated intensive courses of aminoglycosides. It is probable that these strains may persist during antibiotic therapy despite their reduced virulence.[146]

A further group of antimicrobial agents is the 4-fluoroquinolones, of which ciprofloxacin is one of the most active. Despite broad spectrum and rapid bactericidal activity, ciprofloxacin-resistant bacteria are isolated particularly amongst *P. aeruginosa* and staphylococci. As ciprofloxacin is available for both oral and parenteral administration, it has been used extensively in the treatment of patients with cystic fibrosis. However, resistant strains of *P. aeruginosa* have been isolated during such treatment. Ravizzola *et al.*[147] examined two routine clinical isolates and their in-vitro ciprofloxacin-resistant variants whose MICs were >200 mg/L and 50 mg/L respectively. These strains were injected intraperitoneally into mice and the LD50s were calculated. The authors concluded that these ciprofloxacin-resistant strains were less virulent than the susceptible parent strains. A change in colony form (from large/rough to small/rough) was observed. The results suggested that resistance may be associated to a decrease in virulence.

In conclusion, since resistance and virulence genes can be harboured on plasmids and, occasionally, on the same plasmid, excessive use of antibiotics may favour (i) the spread of such plasmids harbouring these two types of genes, and (ii) the formation of such plasmids harbouring the two kinds of markers. Conversely, bacteria exhibiting changes usually associated with chromosomal mutations showed a reduction in virulence and infectivity but rarely a change in immunogenicity. Clearly agents able to select such resistance mechanisms should be used cautiously in circumstances where such mutations may be readily expressed because, although the strains appear to be less virulent, they are still resistant to therapy. Nevertheless, these chromosomally resistant mutant strains may be useful in the development of new bacterial vaccines in the light of their immunogenic but non-pathogenic properties.

SENSITIVITY OF ANTIBIOTIC RESISTANT BACTERIA TO OPSONOPHAGOCYTOSIS *IN VITRO*: IMPLICATIONS FOR CLINICAL INFECTIONS AND THERAPY

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Since the advent of penicillin in 1940 and its subsequent use in the treatment of staphylococcal and other infections, concurrent resistance to the drug has been recognised amongst a hitherto susceptible bacterial population. In particular, the development of methicillin as a therapeutic drug followed the recognition that *Staphylococcus aureus* readily developed resistance to penicillin by the induction of a β-lactamase enzyme. Stereochemical features of methicillin prevented destruction of the antibiotic by the enzyme. However, resistance to this drug is now possible through alteration in the drug target proteins and methicillin-resistant *S. aureus* (MRSA) are now a clinical problem world-wide.[148] Cross-resistance to other penicillins and cephalosporins, as well as resistance to drugs with different biochemical actions, is now found amongst this group of organisms.

MRSA strains are important nosocomial pathogens. They were first described in the early 1960s and outbreaks of MRSA infection were described subsequently in Great Britain, Europe, the USA and Australia. With the exception of a single strain causing clinical problems in London and the South-East of England (named EMRSA-1)[149] most outbreaks of infection with MRSA have been readily contained. This has led to the suggestion that such strains are generally less virulent than their methicillin-sensitive counterparts (MSSA). Isolates of *S. aureus* from patients with primary staphylococcal sepsis are still rarely multi-resistant whereas those patients with MRSA usually have underlying disease, e.g., MRSA strains isolated from patients in a Burns Unit succeeded in colonising the most severely burned individuals but rarely spread elsewhere in the hospital.[150] In terms of patient management, it would be useful to be able to distinguish strains that are potentially

epidemic from non-epidemic or sporadic strains. One study has shown that production of low levels of protein A and high levels of coagulase may be predictive of potentially epidemic strains.[151] Some corroboration of these findings has been shown by others, who also demonstrated production of similar amounts of α, γ and δ haemolysins in MRSA and MSSA.[152]

With this background, it seemed logical to look for differences in host response to infection as a predictor of susceptibility to infection by MRSA. Measurement of various parameters of neutrophil function *in vitro* is possible and by so doing the expression or otherwise or surface components inimical to opsonophagocytosis might be detected. In addition, it is possible to compare neutrophil functions in normal and immunocompromised hosts.

Since 1985, the Royal Infirmary, Glasgow, has witnessed a succession of multi-resistant *S. aureus* strains with differing antibiotic susceptibility patterns. Patients admitted to the Burns Unit became colonised with a strain resistant to penicillin, erythromycin and fucidin (PEF). Sometime thereafter, a strain resistant to penicillin, erythromycin, methicillin and gentamicin (PEMG) appeared in these patients to the exclusion of the former strain. Strains with different antibiotic sensitivity patterns were chosen for measurement of their susceptibility to serum opsonisation and subsequent phagocytosis by neutrophils. However, no significant differences between the strains were found.[153] Similar results have been reported also by others.[152] suggesting that the observed changes in protein A expression are not in themselves sufficient to alter bacterial susceptibility to phagocytosis or that some compensation has taken place in the MRSA strains deficient in protein A.

Extension of these methods to neutrophils obtained from immunocompromised (i.e., burned) patients revealed that the host status was important in determining how MRSA were handled. Immediately after burn injury, patients' neutrophils were markedly depressed in terms of their ability to phagocytose MRSA and this change lasted for several days before normal phagocytic efficacy returned. To some extent, these differences could be attributed to difference in the opsonic activity of the patients' serum (table XI). Therefore, these findings would support the concept that drug resistance *per se* does not predetermine virulence *in vivo* except amongst immunocompromised patients, in whom humoral and cellular defects can be recognised.

It is realised that the drug-resistant *S. aureus* strains used in the preceding studies may or may not have had a common genetic background and cannot, therefore, be properly compared to each other or to antibiotic-sensitive strains. In an attempt to answer this criticism, a different approach has been followed with regard to the development of fluoroquinolone resistance in *S. aureus* and *Pseudomonas aeruginosa*.

The primary action of the quinolones is on DNA gyrase, although the cidal mechanism may involve a series of events.[154] Resistance to drugs of this type is based upon the possession of an altered DNA gyrase, with the A sub-units being intimately involved. Mutations affecting synthesis of a particular outer membrane protein and permeability have been described also. Many of these mutant strains have appeared during therapy and have constituted a risk to the patient as well as a threat to the choice of the quinolones in therapy.

Serial subculture of many bacterial species in the presence of sub-inhibitory concentrations of norfloxacin, ciprofloxacin, ofloxacin, enoxacin or pefloxacin results in the selection of resistance; *Escherichia coli* may be an exception. Cross-resistance between the five drugs was readily demonstrable.[155] This technique has been used to provide quinolone-resistant variants derived from an original quinolone-sensitive strain as targets for measurement of their comparative susceptibility to phagocytosis. Ciprofloxacin and ofloxacin were used as selector drugs. No differences were recognisable in ingestion by neutrophils between the quinolone-resistant offspring and the *S. aureus* or *P. aeruginosa* parent strains.[156] However, when their ability to induce a respiratory burst in the neutrophils was measured, some differences were found between the strains. Variants of *S. aureus* expressing low or intermediate level resistance to the quinolones were less able to generate a respiratory burst than the original strain. In contrast, similar strains of *P. aeruginosa* appeared more capable of stimulating the neutrophils in this assay. In addition, there was considerable strain-to-strain variation in neutrophil interaction which could not be explained either by their innate susceptibility to phagocytic ingestion or their level of drug resistance. Table XII illustrates some of these results. It remains to be seen whether the drug-induced variants differ in other respects from their parent strains. Preliminary evidence with *S. aureus* suggests that their ability to elaborate various exotoxins (e.g., α-haemolysin, coagulase) may be altered. Whether their structural topography is also modified during the development of drug resistance is still to be investigated.

On the basis of current experimental approaches there is little evidence to suggest that development of drug resistance results in "superbugs" capable of initiating more serious infections than their drug-sensitive counterparts, except, perhaps, in causing nosocomial infection among immunocompromised patients. In this context, it would be appropriate to extend the studies to bacterial pathogens in which drug resistance has developed during antibiotic therapy. The selective pressure provided by the antibiotic ciprofloxacin used in the treatment of peritonitis in patients undergoing chronic ambulatory peritoneal dialysis has provided drug-sensitive and drug-resistant strains of *S. epidermidis* and *S. haemolyticus*. However, no differences in bacterial susceptibility to opsonophagocytosis were detected.

THE CLINICAL IMPLICATIONS OF ACQUIRED BACTERIAL RESISTANCE

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The general implications of acquired bacterial resistance to antimicrobial agents are very great. The explosive growth of a new industry of bacterial genetics has been given a

Table XI. Differences between serum from burned patients A and B and normal serum in opsonisation of strains of *S. aureus*

Target strain	Percentage ingested by PMNL after opsonisation with normal serum		patient A serum		patient B serum	
	1%	10%	1%	10%	1%	10%
PRF	12·5	23·1	3·9	10·8	1·6	3·6
PEMG	17·2	22·5	13·2	25·2	1·0	9·5

Table XII. Induction of repiratory burst in PMNL following exposure to quinolone-sensitive and quinolone-resistant strains of *S. aureus*

Strain	Drug resistance	Peak chemiluminescence (cpsc) after opsonisation with serum	
		1%	10%
BRO_1	None	0·27	0·27
	Ciprofloxacin	0·19	0·36
	Ofloxacin	0·41	0·45
LH_4	None	0·40	0·36
	Ciprofloxacin	0·24	0·35
	Ofloxacin	0·10	0·19

powerful impetus by the stimulus of enquiry into the mechanisms of acquired bacterial resistance and has generated considerable activity in academic departments of medical microbiology and research laboratories. A second consequence has been to make medical microbiologists much more "clinical" and much less laboratory based. In the UK, there are approximately 400 consultant medical microbiologists with an infrastructure of junior medical staff, specialised laboratory staff and whole sections of laboratories, for the basis of development has been the existence of antibiotics and the linked relationship with antibiotic resistance. Before the antibiotic era there were hardly any specialist microbiologists in hospitals in the UK; there were relatively small numbers in University departments whose functions were related to teaching and research.

The development of antibiotic resistance has added greatly to the interest of medical microbiology and has provided opportunities for international links, often through the support of the pharmaceutical industry for conferences at home and abroad. The development of the antimicrobial part of the pharmaceutical industry has been very striking. Had there been no need to develop this industry because bacteria had not acquired resistance, there would be very different patterns of prescribing within hospitals, where one fifth of the drugs budget is now allocated to antimicrobial agents, and a corresponding reduction in the interest of the industry in microbiologists.

All drugs have certain common limitations on their use—the nature and frequency of side effects, and cost. Antimicrobial agents have a third problem which has generated the antibiotics industry and medical microbiology as a speciality—acquired bacterial resistance to antibiotics.

Selection of resistant organisms

In the early days of the antimicrobial era, most infections were caused by organisms, such as staphylococci, streptococci, and, very occasionally, coliform organisms, which were inherently antibiotic-sensitive. Over subsequent decades, selection pressures produced by the widespread use of antibiotics, whether for appropriate or inappropriate reasons, have resulted in the emergence of new groups of major pathogens as causes of infections. Often this has led to the emergence of organisms of which microbioligists were barely aware before the advent of antimicrobial agents; infections caused by *Pseudomonas aeruginosa,* and even by other *Pseudomonas* spp., by *Klebsiella, Enterobacter, Serratia, Arizona* and *Citrobacter* spp. and by many other gram-negative species, by enterococci, and by resistant staphylococci, whether coagulase-negative or coagulase-positive, are now common. The pressures for the emergence of these oragnisms as pathogens have been, on the one hand technological change, such as the increasingly widespread use of indwelling vascular catheters or prosthetic devices, or more heroic surgical procedures, and, on the other hand, the attempt to prevent these ever more exposed patients succumbing to infections by the widespread use of antibiotics, whether given prophylactically or therapeutically.

New drugs

In the classical era of the development of new antimicrobial agents, extending to the late 1950s, although antibiotic resistance was emerging it has not done so to any great extent and several powerful agents were available that covered nearly all the pathogens that were commonly seen in clinical practice at that time; penicillin, streptomycin, tetracycline, chloramphenicol, sulphonamide, erythromycin, ampicillin, carbenicillin, methicillin, the first generation cephalosporins and metronidazole were all available.

However, many of those drugs, useful though they were, had limitations on their use because of difficulty of administration or, in cases such as streptomycin, toxicity. There would, perhaps, have been some development of new drugs even in the absence of bacterial resistance, as attempts to develop more convenient versions of existing drugs which would have been easier to administer and perhaps less toxic. It is reasonable to speculate that there would not have been the development of the semi-synthetic β-lactam agents—the cephalosporins, monobactams, augmentin, etc—or of trimethoprim and co-trimoxazole, and that the massive development of the aminoglycosides, and the quinolones and glycopeptide antibiotics, would not have occurred without the major stimulus of bacterial resistance. If it were not for the problem of resistance, most of these relatively new antimicrobial agents would have offered little to improve patient management.

Implications for patients

The response of the medical profession to the problem of resistance has been to use higher doses of the older drugs than would otherwise have been the case, or to combine drugs, as in the treatment of tuberculosis, to overcome the actual or perceived risk of resistance to single agents, or, alternatively, to use more recently developed drugs which may be difficult to administer, toxic and costly.

Some drugs are difficult to administer because they have to be given intravenously, commonly in substantial doses, generally necessitating the positioning of long intravenous catheters, which essentially ties the patient to a hospital bed and makes him or her immobile; there is also the administration, in some cases, of a heavy sodium load. There are well documented risks of morbidity and mortality relating to the length of time for which an intravenous catheter has to remain in place.

Toxicity and side effects present considerable problems in relation to monitoring levels of the agents in serum and the cost of this monitoring, both in terms of laboratory costs and

in terms of potential litigation when patients have semi-predictable side effects related either to unusual sensitivity to the drug or to long administration of the drug for good clinical reasons. The cost of monitoring and of litigation is substantially under-estimated by the medical profession. The direct cost of the drug also may be very considerable. Pencillin G, 600 mg given 6 hourly, costs a few pence per day. The cost of replacing penicillin G by alternatives such as vancomycin, cephalosporins, etc., may be tens of pounds per day, with the result that excess money spent on the new drugs cannot be spent on more desirable objectives. There are also associated costs in giving drugs, both for the giving system and for the labour of administering the treatment.

Examples of current clinical problems

A recent problem concerns a 6-month-old child with meningitis caused by a pneumococcus with diminished sensitivity to penicillin. Initially, penicillin was given, before it was recognised that the pneumococcus was not fully sensitive, and there was no significant clinical response. Once the problem was recognised, chloramphenicol treatment was instituted but this has caused considerable anxiety because of the need to give a potentially toxic drug to a child who would otherwise not have received it.

In another specialty, cardiac surgeons have used β-lactam antibiotics combined with an aminoglycoside, prophylactically, to reduce post-operative sternal would infection. Such infections are most commonly caused by coagulase-negative staphylococci, which are resistant to methicillin in 60% of cases, or by *Staphylococcus aureus*. Because even very short courses of aminoglycosides given prophylactically may give rise to toxicity, there is increasing pressure to use glycopeptide antibiotics prophylactically.

Doomwatch

There is steadily progressive erosion of the usefulness of antibiotics by rising tide of acquired resistance in pathogenic bacteria. Total inability to control resistant bacteria is, so far, a rarity. However, doctors being driven to use second- or third-line drugs, or antibiotic combinations, rather than the first choices of previous years. A heavy cost in side effects, ecological damage, preventable hospitalisation, morbidity and mortality, and in money, is being paid and will increasingly be paid.

Concluding remarks by J. T. Smith

In the "good old days", a single lecture describing bacterial resistance to antibacterial agents would nicely encompass all there was to know on the subject. However, as clinical isolates exhibiting resistance to more and more drugs became more frequent (more often than not resulting from R-plasmids), whole conferences on resistance to but a single class of antibacterial agent became normal. Although such a fashion had technical merit, discussion of bacterial resistance became so fragmented that few indeed could retain an overview of the problem. The meeting that provided the basis for this review article brought together investigators with a wide range of expertise across the full spectrum of antibiotic resistance and helped speakers and audience alike to stay abreast of this most important topic.

We thank Bayer UK for supporting the meeting on which this article is based, which has permitted a forum where problems of antibiotic resistance could be frankly discussed.

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Comparison of *Clostridium sordellii* toxins HT and LT with toxins A and B of *C. difficile*

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Summary. *Clostridium sordellii* produces two toxins, designated HT (haemorrhagic toxin) and LT (lethal toxin), that are similar to toxins A and B of *C. difficile*. The physicochemical properties of toxins HT and A were remarkably similar. The specific biological activities of toxin HT were almost the same as those of toxin A, and their NH_2-terminal sequences shared close homology. The properties of toxins LT and B were similar, as were their NH_2-terminal sequences, but toxin B was much more cytotoxic than toxin LT. Immunodiffusion analysis with specific antibodies showed that although toxins B and LT shared major antigenic determinants, each had unique epitopes. The results suggest that toxins B and LT have diverged more than toxins A and HT. Immunoblotting with antibodies to the toxins of *C. difficile* showed that toxins HT and LT had common antigenic determinants.

Introduction

Several studies have implicated *Clostridium sordellii* as a cause of diarrhoea and enterotoxaemia in domestic animals,[1–3] and, more recently, as an agent of toxic shock-like syndrome in man.[4,5] This species was once suspected to be the cause of pseudomembranous colitis (PMC) in man because the cytotoxicity of faecal filtrates from PMC patients was neutralised by *C. sordellii* antitoxin.[6–8] However, *C. sordellii* could not be isolated from the faeces of patients with PMC. This discrepancy was clarified when *C. difficile* was isolated from faecal samples of patients with PMC,[9–11] and it was shown that the toxins produced by this organism are neutralised by *C. sordellii* antitoxin.[6,8,12,13] Two toxins, A and B, have been purified from culture supernate of toxigenic strains of *C. difficile*.[14–16] Both are large proteins that are lethal to animals and cytotoxic. Toxin A is a potent enterotoxin that produces a haemorrhagic fluid response in the rabbit ileal loop assay.[17–19]

C. sordellii produces two toxins that are similar to toxins A and B, which explains why *C. sordellii* antitoxin neutralises the toxins of *C. difficile*. The production of two distinct toxins by *C. sordellii* was first described by Arseculeratne *et al.*,[20] who extracted a haemorrhagic toxin from sporulating cells and an oedema-producing toxin from vegetative cells. The oedema-producing toxin was more lethal than the haemorrhagic toxin and the toxins are now referred to as LT (lethal toxin) and HT (haemorrhagic toxin) respectively. We have already described the purification of toxin HT by ultrafiltration and immuno-affinity chromatography with a monoclonal antibody (MAb) to toxin A, and have shown that toxin HT has biological activities and immunological properties similar to those of toxin A.[21] Popoff has purified toxin LT and shown that it is immunologically related to toxin B.[22] The toxins produced by *C. difficile* and by *C. sordellii* have similar physicochemical as well as immunological and biological properties but they are not identical. Therefore, it was of interest to compare the properties of these toxins in more detail.

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Materials and methods

Protein determination

Protein concentration was estimated by the method of Bradford[23] with the BioRad Protein Assay Kit (BioRad Laboratories, Richmond, CA, USA). Bovine γ-globulin (BioRad) was the standard.

Bacterial strains and medium

C. difficile VPI 10463 (Tox^+), *C. sordellii* VPI 9048 (Tox^+), VPI 2013 (Tox^-) and VPI 7319 (Tox^-) were obtained from the culture collection of the Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University (Blacksburg) and were identified by L. V. Holdeman, E. P. Cato and W. E. C. Moore by methods described in the Virginia Polytechnic Institute Anaerobe Laboratory Manual.[24]

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DOES CLAVULANIC ACID SELECT EXTENDED-SPECTRUM β-LACTAMASES?

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ABSTRACT

Escherichia coli J62-2 (TEM-5_{pCFF14}) was cultured in liquid media containing limiting concentrations of ampicillin or amoxycillin and clavulanic acid (2:1 ratio). The cultures were subcultured five times into fresh media containing the same antibiotics. Ten colonies were purified from each subculture (300 strains in total). Minimum inhibitory concentrations (MICs) of ampicillin, amoxycillin, ceftazidime, cefotaxime, and amoxycillin/clavulanic acid revealed a few strains with increased resistance to not only amoxycillin/clavulanic acid but also ceftazidime and cefotaxime. Isoelectric focusing demonstrated that the increased resistance corresponded with multiple β-lactamase expression (pI 5.57, 5.5, and 5.25). Electrophoresis of plasmid DNA confirmed that each organism contained a single plasmid, the same size as pCFF14 (150-kb). Analysis of enzyme kinetics of each β-lactamase (pI 5.57, 5.5, and 5.25) compared to TEM-1 (pI 5.4), TEM-5 (pI 5.5), TEM-10 (pI 5.57), and TEM-12 (pI 5.25) suggests that the unknown β-lactamases are TEM-5, TEM-10 and TEM-12 respectively. Our results suggest that amoxycillin in combination with clavulanic acid selects back mutations to TEM-1 and other extended-spectrum β-lactamases. The host organism will contain a heterogenous population of a clinical plasmid.

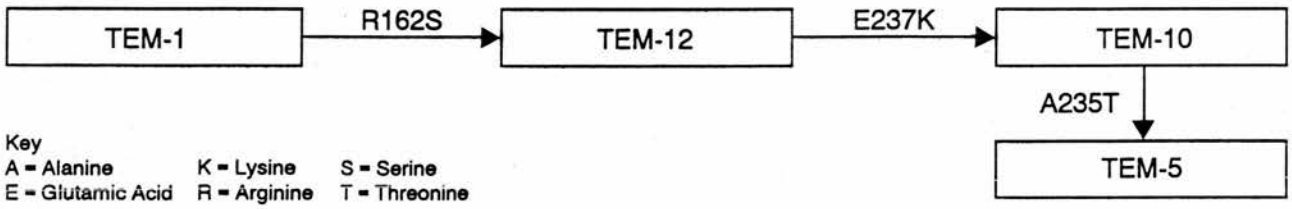
INTRODUCTION

The third-generation cephalosporins (3GCs), which possess a 7-oxime side-chain, epitomise our ability to overcome antimicrobial resistance, especially of the plasmid-encoded TEM-1 and SHV-1 β-lactamases. Unfortunately the TEM- and SHV-derived extended-spectrum β-lactamases (ESBL) have developed as the direct result of the selective pressure imposed by the use of 3GCs.

The biochemical data published by Sowek and colleagues in 1991 [1] show that as cefotaxime and ceftazidime turnover, k_{CAT}, increases, the benzylpenicillin turnover decreases. The greater the efficiency of 3GC hydrolysis the slower the turnover of benzylpenicillin.

When considering the mutations required to transform TEM-1 into TEM-5 (Figure 1): comparison of the benzylpenicillin turnover number of TEM-12 to that of TEM-10 shows that, although the latter enzyme hydrolyses the 3GCs with greater efficiency than the former, TEM-12 hydrolyses penicillins faster than TEM-10. Biological pressure from a penicillin would select against the ESBL and may induce back mutations to the more innocuous enzyme, TEM-12. We have proposed the hypothesis that the evolution of the parent β-lactamase to broad-spectrum enzyme can consist of both forward and backward mutations at well defined locations.

FIGURE 1



METHODS

The passage experiment methodology was a modification of that by described by Thomson & Amyes [2]: *Escherichia coli* J62-2 (TEM-5_{pCFF14}) was cultured in liquid media containing limiting concentrations of ampicillin (AMP-selection) and amoxycillin and clavulanic acid (2:1 ratio) (CO-AMOXICLAV-selection). The cultures were subcultured five times into fresh media containing the same antibiotic. Ten colonies were purified from each subculture (300 strains in total).

Minimum inhibitory concentrations (MICs) of ampicillin, amoxycillin, ceftazidime, cefotaxime, and amoxycillin/clavulanic acid were performed according to the BSAC guidelines [3].

Isoelectric focusing (IEF), based on the method of Matthews *et al* [4], employing a broad-range ampholine mixture (pH 3.5-10) was used to examine a selection of the β-lactamases produced.

The method of Takahashi & Nagano [5] was used to extract and visualise the plasmid DNA of those strains examined by IEF. The plasmid DNA was also digested with a number of restriction endonucleases, none of which were known to cut within the TEM β-lactamase gene. Southern Blots were prepared according to the method of Southern [6] and were subsequently analysed by hybridisation with a non-radioactively labelled *bla*T-1 probe (generated by PCR and Biotin-7-dATP) [7].

β-lactamase activity and the effect of clavulanic acid inhibition were determined by spectrophotometric assays [8, 9, 10].

RESULTS

MICs of the colonies from the AMPICILLIN-selection group revealed a few strains with increased resistance to all of the antibiotics (ampicillin, amoxycillin, ceftazidime, cefotaxime, and amoxycillin/clavulanic acid) used in the challenge. Isoelectric focusing demonstrated that all the β-lactamases were identical and had an isoelectric point (pI) of 5.5 corresponding to that of TEM-5. Specific activity of the crude enzyme preparations suggested that enzyme production in the cell was raised. No β-lactamase mutations were observed as a result of the ampicillin.

MICs of colonies from the CO-AMOXICLAV-selection to ampicillin, amoxycillin, ceftazidime, cefotaxime, and amoxycillin/clavulanic acid revealed a few strains with increased resistance not only to amoxycillin/clavulanic acid but also ceftazidime and cefotaxime. Isoelectric focusing demonstrated that the increased resistance corresponded with multiple β-lactamase expression (pI 5.57, 5.5, and 5.25). Electrophoresis of plasmid DNA confirmed that each organism contained only a single plasmid of similar size and *Eco*R I restriction pattern to pCFF14 (150-kb).

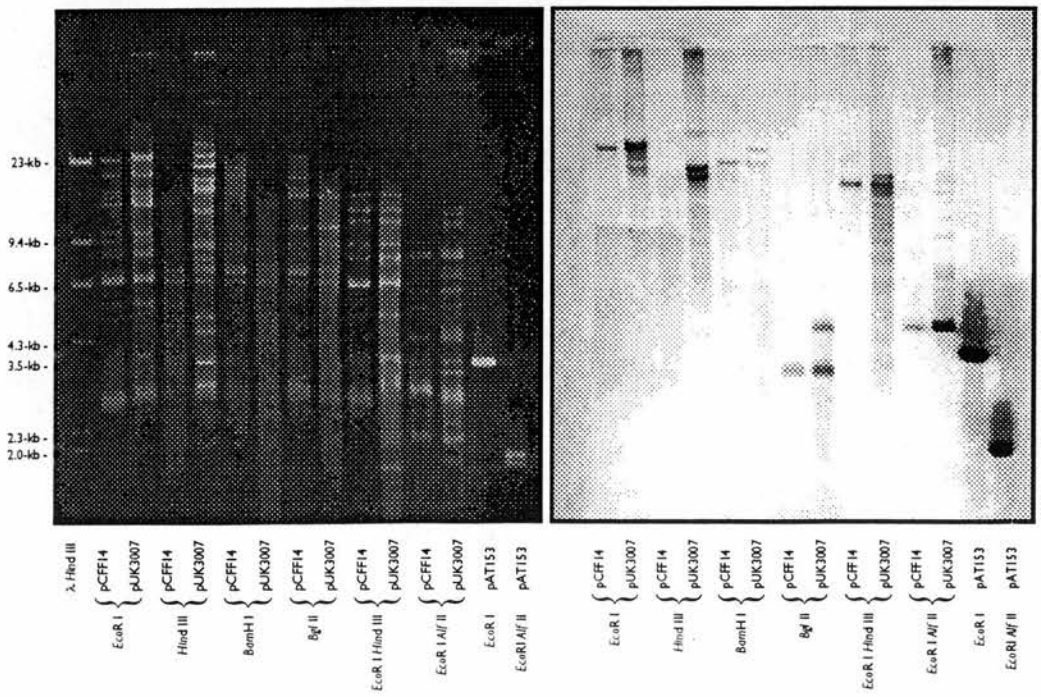
Comparison of the restriction patterns of pCFF14 and one of the mutant strain's plasmid (named pUK3007) revealed an additional *Eco*R I restriction fragment, approximately 25-30-kb in size. Additional restriction fragments are seen in the restriction patterns with some of the other endonucleases used.

Intragenic gene probing revealed a *bla*T gene was only ever present on a single restriction fragment from a digest of pCFF14. However, a TEM gene was shown to be present on two restriction fragments of many of the pUK3007 digests.

Specific activity of the crude enzyme preparations of TEM-5_{pCFF14} and the enzyme(s) of pUK3007 suggested that the latter preparation possessed a much greater spectrum of activity than would be exhibited by a single enzyme, TEM-1, TEM-12, TEM-10, or TEM-5. This is in agreement with the IEF results showing the presence of more than one TEM β-lactamase.

Our results show that ampicillin does not induce point-mutations in the ESBL. However, the use of amoxycillin and clavulanic acid in combination causes gene duplication and point mutations in the *bla* genes to occur in the clinical plasmid, pCFF14. The resulting plasmid exhibits the production of three β-lactamases: TEM-5, TEM-10, and TEM-12. TEM-10 and TEM-12 are one and two point mutations different from TEM-5 respectively.

FIGURE 2



MIC'S OF β-LACTAMS & HYDROLYTIC CHARACTERISTICS OF TEM-DERIVED β-LACTAMASES

Enzyme	Benzylpenicillin		Ceftazidime		Cefotaxime	
	MIC*	k_{CAT} (s^{-1})	MIC*	k_{CAT} (s^{-1})	MIC*	k_{CAT} (s^{-1})
TEM-1	>128	520	0.25	0.0016	0.03	0.25
TEM-12	>128	54	2.0	3.4	0.03	2.4
TEM-10	>128	17	32	17	0.25	0.39
TEM-5	-	-	128	-	4	-

* Minimum inhibitory concentration in μg/ml for plasmid-containing *Escherichia coli*

DISCUSSION AND CONCLUSIONS

Conservative mutations or mutations remote from the active site do not affect biological parameters in a measurable manner. Evolutionary implications of the single mutations may be relatively minor and may not be detected during the normal course of events. Silent mutations, or minimally important mutations, occur frequently with little functional consequence but may prepare bacteria to be ready for a more drastic change when challenged with a 3GC. Acquisition of a single innocuous mutation facilitates the occurrence a more dangerous double mutation.

Although innocuous mutations may exist in the unchallenged population, treatment with a penicillin, as often occurs in general practice, would result in the selection of the parental enzymes, TEM-1, TEM-2 and SHV-1, rather than the progeny. Challenge with the 3GCs would select the most resistant β-lactamase expressed in the bacterial flora. Only when such an event has occurred will a second mutation event occur to expand the spectrum of the enzyme further. A third mutation may occur during continued treatment with 3GCs to expand the spectrum of the β-lactamase further.

Cross-infection to other patients or escape into the environment may result in the challenge of the host organism with a penicillin **and a β-lactamase inhibitor.** Such a biological pressure would select against the extended-spectrum β-lactamase and may induce back mutations to the more innocuous enzymes, TEM-17 or SHV-3 for example.

We suggest the hypothesis that the evolution of the parent β-lactamase to broad-spectrum enzyme can consist of both forward and backward mutations at well defined locations.

Our results suggest that amoxycillin in combination with clavulanic acid selects back mutations to TEM-1 and other extended-spectrum β-lactamases. The back mutations are facilitated by gene duplication. The host organism will also contain a heterogenous population of a clinical plasmid. We believe that point-mutations occur in the TEM-gene during duplication of the gene. In a non-selective environment one of the duplicate genes is usually deleted. However, the results of this experiment show that the selective pressure which results from the combination of a penicillin, such as amoxycillin, with clavulanic acid preserves the presence of duplicate genes on the same plasmid.

The acquistion of more than one ESBL by the cell broadens the resistance profile of the bacterium allowing significant 3GC, penicillin, and β-lactamase inhibitor resistance. A subsequent challenge with amoxycillin/ clavulanic acid may promote further back-mutation towards TEM-1, away from ESBL activity. However, the use of a 3GC may select novel ESBL, not necessarily the ancestral ESBL.

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